PP
1
J88
v.153
no.3

PR

THE JOURNAL OF PHYSIOLOGY

THE UNIVERSITY OF MICHIGAN
NOV 7 1960
SCIENCE LIBRARY

EDITED FOR

THE PHYSIOLOGICAL SOCIETY

BY

N. AMBACHE
P. M. DANIEL
G. W. HARRIS
R. J. LINDEN
R. V. COXON
S. P. DATTA
R. C. JORDAN
H. R. NOLTIE
M. DE B. DALY
G. GORDON
B. KATZ
W. J. O'CONNOR
W. D. M. PATON
G. S. BRINDLEY
(*Secretary*)
W. A. H. RUSHTON
R. D. KEYNES
(*Chairman*)

October 1960
Volume 153, No. 3

CAMBRIDGE UNIVERSITY PRESS
Bentley House, 200 Euston Road, London, N.W. 1
American Branch: 32 East 57th Street, New York 22, N.Y.

Price 30s. net
(U.S.A. $5.00)

J. Physiol. (1960), **153**, *pp.* 413–422
With 4 text-figures
Printed in Great Britain

CHANGES IN pH OF THE PERFUSATE DURING HYPOXIA IN ISOLATED PERFUSED CAT LUNGS

BY HELEN N. DUKE, THE LATE ESTHER M. KILLICK AND JEAN V. MARCHANT

From the Department of Physiology, Royal Free Hospital School of Medicine, London, W.C. 1

(*Received for publication:* 17 *February* 1960)

Hypoxia has been shown to produce an increased pulmonary arterial pressure in the anaesthetized cat (von Euler & Liljestrand, 1946; Logaras, 1947). An increase in pulmonary vascular resistance also occurs in isolated perfused cat lungs (Nisell, 1948; Duke, 1951, 1954, 1957; Duke & Killick, 1952) and in the perfused left lung preparation of the anaesthetized cat (Duke, 1954, 1957) in response to substituting a gas mixture of low oxygen content for air. The experiments now reported were performed in order to test the hypothesis (Liljestrand, 1958) that hypoxia acts on the pulmonary blood vessels by liberation of lactic acid and thereby may cause a local regulation of the relation between blood flow and ventilation.

METHODS

Isolated cat lungs were set up and perfused through the pulmonary artery at constant-volume inflow as previously described (Duke, 1951). Gas mixtures used for ventilation were made by the British Oxygen Company and were contained in Douglas bags which were attached to the pump. The lungs were ventilated either with air or with N_2. The period during which the lungs were exposed to hypoxia varied between 3 and 25 min but was usually 10–15 min. In one experiment 5 % O_2 in N_2 was used instead of N_2. In three experiments 7 % CO_2 in air was used instead of air and the change was made to 7 % CO_2 in N_2; in the remaining five experiments the change was made from air to N_2.

The initial volume of the perfusate varied between 104 and 160 ml. In order to obtain enough perfusate to allow for taking numerous blood samples additions had to be made to the blood collected from the animal. In two experiments heparinized blood from a donor cat was used, in two other experiments Ringer–Locke solution 25 ml. was added to 79 or 98 ml. blood. In three experiments 30 ml. Dextran (Intradex, Glaxo) was added to the blood (110–120 ml.) before starting the experiments. The pump output varied from 44 to 83 ml./kg body wt./min in different experiments and the total volume of the perfusate would have made a complete circuit at times varying from 30 sec to 1 min 30 sec in different experiments. This time is slightly underestimated because no allowance could be made for the initial volume of blood in the lungs.

Mean pulmonary arterial pressure was measured with a manometer filled with 0·9 % NaCl solution, the zero of which was arranged to be approximately 1 cm posterior to the mitral valve. The pressure was recorded with a small-capacity tambour. Changes in lung blood volume were measured by recording changes in the volume of blood in the venous reservoir.

Under the conditions of these experiments this record shows inverse changes in lung blood volume (Daly, 1938).

Samples of blood were drawn anaerobically from the tubing leading from the left auricle. Lactate, pH, O_2 content, and haemoglobin were determined in these samples with as little delay as possible.

Lactate was determined by the method of Barker & Summerson (1941); ten determinations by this method on a single sample of blood showed a standard deviation of 1·0 mg from the mean percentage value. Total haemoglobin was determined spectrophotometrically; 0·05 ml. blood was mixed with 10 ml. 0·1 % Na_2CO_3 solution, and moist coal gas was bubbled through the mixture. The percentage transmission of this solution of carboxyhaemoglobin was read at a wave-length of 569 mμ (Hunter, 1951). The O_2 content of the blood was determined by the micro-method of Scholander & Roughton (1943).

Blood pH was determined at 37° C with the micro-electrode described by Joels & MacNaughton (1957), using either a Marconi pH meter, or a direct reading meter made by Electronic Instruments. The determination was usually made within 5 min of taking the sample, but during one experiment this was not possible owing to the necessity of checking drift on the pH meter; the maximum delay caused was 30 min and the samples were kept at room temperature during this time. The change in pH under these conditions was negligible; pH determinations made on a sample of heparinized cat blood at 10 min intervals confirmed that the earliest detectable change occurred 40 min after the sample was taken. It is difficult to assess the error of the method of pH determination on blood samples, but from repeated determinations of the pH of standard buffer solutions the error appeared to be $\pm 0{\cdot}02$.

Blood oxygen tension was calculated from O_2 content, O_2 capacity and O_2 saturation of blood samples by using dissociation curves for cat's blood (Duke & Stedeford, 1959).

Sodium bicarbonate and lactic acid solution were injected into the tubing between the left auricle and venous reservoir.

RESULTS

Response to hypoxia

Pulmonary arterial pressure. In twenty-one tests out of twenty-two the pulmonary arterial pressure (PAP), increased when the lungs were ventilated with N_2 instead of air or 7 % CO_2 in N_2 instead of 7 % CO_2 in air. In one test the PAP was unchanged (Fig. 1). The maximum pressure rise was 75 % of the initial value but increases of about 30 % were the most usual. In most, but not all, of the tests the pressure had reached a new stable level before the end of the hypoxic period. It was noticed that in most experiments the magnitude of the pressor response to hypoxia increased with time during the experiment. The one test in which no response occurred was the first test of an experiment in which pressor responses of average size were later obtained. In six tests the pressor responses to hypoxia occurred in the absence of changes in tidal air overflow volume. In the other tests (one of which is shown in Fig. 2) changes of from $+3{\cdot}0$ ml. to $-2{\cdot}5$ ml. were observed.

Blood pH. In fifteen tests out of twenty-two the pH of the left auricular blood was increased during hypoxia, in six of the tests the change in pH was within the limits of error of the determination ($\pm 0{\cdot}02$) and in one it decreased (see Fig. 1). There was apparently no relationship between the

magnitude of the pressor response in different preparations and the change in blood pH. Nor was there any clear indication that in the same preparation the percentage change in pressure was related to the degree of change in blood pH.

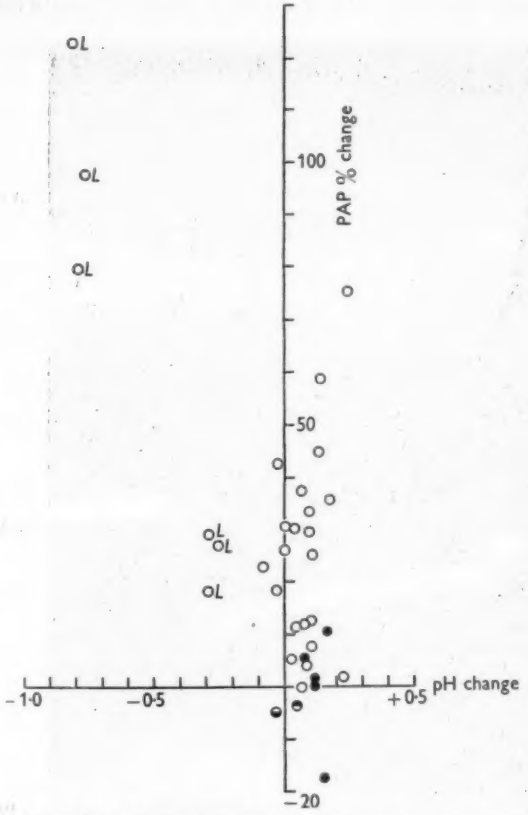


Fig. 1. The relationship between percentage change in pulmonary arterial pressure (ordinate) and change in pH of the left auricular blood (abscissa). Changes produced ○ at the end of a period of hypoxia; ○L after addition of lactic acid; ● after addition of sodium bicarbonate; ◒ during inhalation of 100 % CO instead of air; ◓ during inhalation of 20 % CO instead of air.

There was also no apparent relationship between the initial pH of the left auricular blood and the magnitude of the pressor response in different preparations (Fig. 3). There was no constant relationship between the pO_2 of the left auricular blood during hypoxia and the change in pH, but in three of the six tests in which there was no significant change in pH the

pO_2 of the perfusate remained relatively high (above 40 mm Hg compared with the usual 10–25 mm Hg).

Lactate. Blood lactate was measured about 1 min before changing from air to N_2 ventilation, just before the end of N_2 ventilation, and again after changing back to air. In a total of nineteen tests the lactate concentration

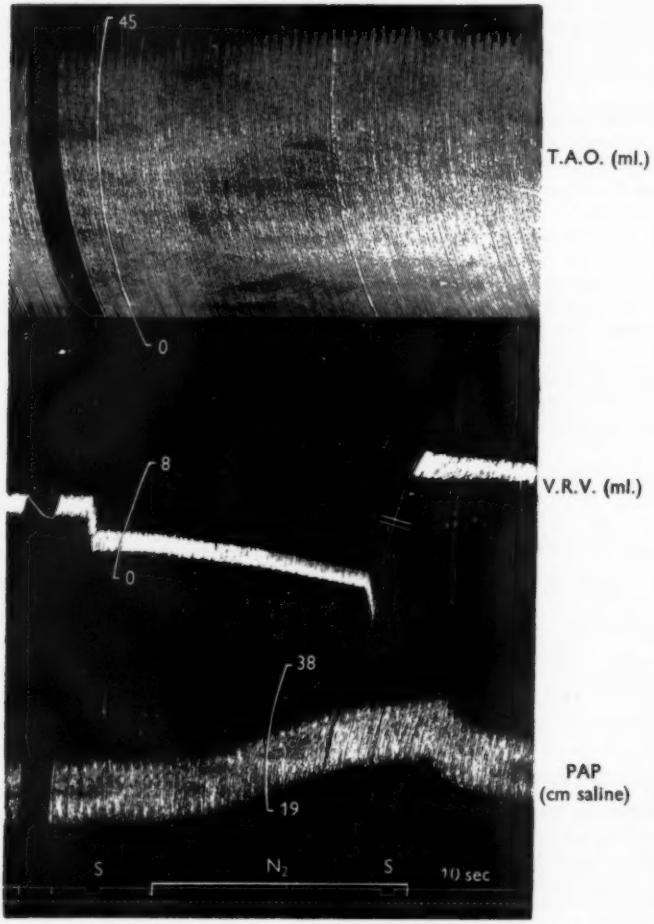


Fig. 2. The effects of changing the ventilating gas mixture from air to N_2. Cat 3·2 kg. Perfusion started at 11.05 a.m. (240 ml./min). During signal (12.22 to 12.29 p.m.) the lungs were ventilated with N_2 instead of air. At S approx. 5 ml. blood taken as sample. T.A.O. = tidal air overflow volume at 7 cm H_2O; V.R.V. = change in venous reservoir volume; PAP = pulmonary arterial pressure. Time marker, 10 sec.

was increased in eight, decreased in five and unchanged in six. The maximum increase in rate of change of lactate concentration during hypoxia compared with the control was 1·6 mg/100 ml./min and the maximum decrease was 1·9 mg/100 ml./min.

It was clear that there was a progressive increase of the blood lactate concentration during each experiment and that any changes due to hypoxia were superimposed on this. This increase in blood lactate was usually rapid in the early stage of perfusion, and became slower as the duration of

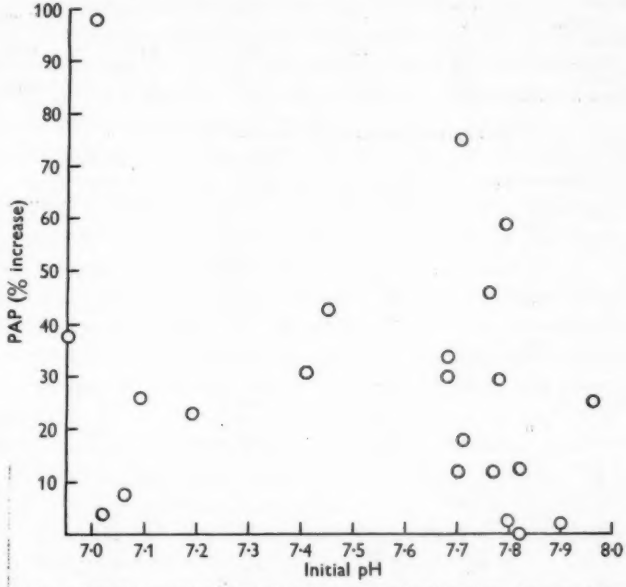


Fig. 3. The effect of the initial pH of the left auricular blood on the percentage increase in pulmonary arterial pressure in response to hypoxia.

perfusion increased. In several experiments it was necessary to add Dextran or Ringer–Locke solution to the perfusate to maintain a sufficient total volume. In other experiments lactic acid was added to the perfusate. Only three experiments provided a long period of perfusion without additions to the perfusate, and the mean rate of lactate increase is shown for these experiments in Table 1 (*a*).

The results for a typical experiment are shown in Fig. 4 in which mean PAP, blood lactate and blood pH are plotted against time. The increasing pulmonary arterial pressor response with time, and the progressive rise in blood lactate can be seen. In this experiment there was an increase of blood pH and a slight increase of blood lactate with hypoxia.

Blood oxygen tension. Immediately before the end of the hypoxic period the pO_2 of the blood from the left auricle was usually 10–25 mm Hg. Since the total volume of fluid in the system was perfused through the

TABLE 1. (*a*) Lactate formation in blood during perfusion through lungs

Perfusate	Amount (ml.)	Duration of perfusion (min)	Lactate (mg/100 ml. blood) Initial value	Final value	Increase (mg/100 ml./min)
Blood + dextran	115 30	144	14·1	50·2	0·25
Blood + donor blood	83 77	191	18·4	61·0	0·22
Blood + donor blood	85 70	116	33·5	92·0	0·50

(*b*) Lactate formation in blood *in vitro* at 37° C

Tonometer gas	Time (min)	Lactate (mg/100 ml. blood)	Lactate increase (mg/100 ml./min)
Air	0	7·5	0·29
	120	42·3	
N_2	146	46·0	0·44
	202	70·5	
Air	207	72·0	0·20
	260	82·7	
Air	0	5·4	0·32
	125	45·2	
N_2	141	46·5	0·24
	197	60·2	
Air	202	60·2	0·21
	231	67·5	

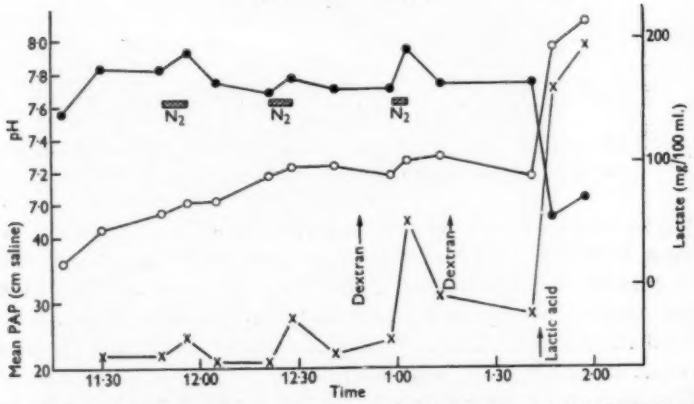


Fig. 4. The effects of hypoxia in isolated perfused cat lungs. Cat 2·5 kg. Blood flow 180 ml./min. Perfusion begun at 11.00 a.m. Positive pressure respiration with air. Periods of N_2 ventilation are indicated. At first two arrows addition of 20 ml. dextran to perfusate; at third arrow addition of 1 ml. N lactic acid to perfusate. ● pH of perfusate; ○ lactate content of perfusate; × mean pulmonary arterial pressure.

lungs in a time varying between 30 sec and 1 min 30 sec in different preparations, it would be expected that the pO_2 of the pulmonary arterial blood would decrease *pari passu* with that of the alveolar air and the left auricular blood. Studies made on other preparations perfused in similar circumstances (H. N. Duke, unpublished observations) have shown that the pO_2 of the pulmonary arterial blood differs little from that of the left auricular blood. There was no apparent relationship between the size of the pressor response to hypoxia and the change in blood pO_2 either in the same preparation at different times or in different preparations.

In twelve tests in which the pO_2 of the left auricular blood was between 10 and 25 mm Hg the percentage increase in PAP varied from 4 to 75; in six tests in which the pO_2 was between 25 and 50 mm Hg, the percentage increase in PAP varied from 12 to 59.

Addition of lactic acid and sodium bicarbonate. In six tests 0·25–1·0 ml. N lactic acid was added to the perfusate in the venous reservoir. This caused a rise in PAP and a reduction of left auricular blood pH (Fig. 1). The increase in PAP was more sustained than during the hypoxic tests, probably because the stimulus could not be withdrawn.

In five tests sodium bicarbonate was added in solution to the venous reservoir in amounts varying from 50 to 100 mg. In three tests the pulmonary arterial pressure was slightly increased, in one test it was decreased and in one test it was unchanged. In all the tests the blood pH was increased (Fig. 1).

Carbon monoxide. In two tests CO was used to ventilate the lungs instead of air. In both tests the PAP was decreased (Fig. 1). With 20% CO in air the blood pH was increased and with 100% CO the blood pH was decreased, although the changes in pH are of doubtful significance, since they are very near to the limits of error of the method.

Lactate accumulation in blood in vitro. In view of the gradual increase in the lactate content of blood during lung perfusion it was decided to investigate changes in blood lactate during rotation of blood in a tonometer at 37° C. Cat blood was used, drawn and heparinized in the same way as for the lung perfusion. Lactate was determined before placing the blood in the tonometer, and subsequent samples were withdrawn for lactate determination at intervals of approximately 30 min. After 2 hr the air in the tonometer was replaced by N_2 and the lactate determinations continued. After another hour the N_2 was replaced with air and lactate determinations again continued. The saturation of the blood with O_2 fell to about 10% when the tonometer contained N_2.

The lactate in the blood increased under these conditions (Table 1 *b*). The change from air to N_2 was associated with an increased rate of rise of lactate in one experiment; in the second experiment it seemed to have

little effect. The rate of increase of lactate *in vitro* was of the same order of magnitude as the rate during lung perfusion.

DISCUSSION

Liljestrand (1958) found that the pulmonary arterial pressure might show either a rise or a fall in response to hypoxia in isolated lungs of cats perfused through the pulmonary artery at constant volume inflow. The former effect was associated with a decrease in pH of the left auricular blood and the latter effect with an increase in blood pH. The results were unaffected by the addition of 5 % CO_2 to both gas mixtures. The results now reported do not confirm this dual response and this accords with previous observations in similar preparations (Duke, 1951, 1954, 1957; Duke & Killick, 1952). Ventilation of the lungs with N_2 or with mixtures containing less than 10 % O_2 caused a rise in pulmonary arterial pressure whether the pulmonary arterial blood was fully saturated with O_2 or whether the pO_2 in this blood was reduced concomitantly with that of the alveolar air by recirculation of the blood (Duke & Killick, 1952; Duke, 1954). The almost invariable finding in the present series of experiments has been an increase of pulmonary arterial pressure in response to hypoxia. This was usually associated with an increase of the left auricular blood pH but the pressor response did not appear to depend either on the initial blood pH or on the extent or direction of the change in blood pH. The extent of the pressor response appeared unrelated to the pO_2 in the left auricular blood, which under the conditions of perfusion differed little from the pO_2 of the pulmonary arterial blood. Addition of 7 % CO_2 to the air or N_2 which was used to ventilate the lungs did not appear to influence the results.

In the present series of experiments the blood used for perfusion was diluted with blood from a donor animal, dextran or Ringer–Locke solution. The latter solution was used by Liljestrand (1958). No difference was noted in the results in consequence of these varying procedures.

The increase in lactate concentration of the perfusate during the course of each experiment was a constant and striking feature of the present experiments. Equilibration of cats' blood with air or N_2 at 37° C for several hours also led to an increase in the concentration of lactate in the blood and the final blood lactate concentration was similar to the concentration reached in the perfusate of isolated lungs after a similar time interval. In one of the tests the concentration of lactate in the blood increased more rapidly when the blood was exposed to N_2 than when it was exposed to air, but in the other test the increase in blood lactate occurred at a similar rate with air and N_2. This is in agreement with the findings in the perfused lungs in that hypoxia of the perfused lungs did not invariably lead to a

rise in the concentration of lactate in the perfusate. The tension of CO_2 in these *in vitro* experiments was not measured but experience with cats' blood in similar circumstances would lead one to believe that the CO_2 tension was 2–4 mm Hg (Duke & Stedeford, 1959).

The rise in blood pH found in the present series of experiments as a result of hypoxia may be associated with the change of oxyhaemoglobin to reduced haemoglobin in the perfusate. Values for the change in pH produced by this are not available for cat blood, but those for horse haemoglobin given by German & Wyman (1937) make this a reasonable explanation. Dill, Daly & Forbes (1937) found the effects of oxygenation of haemoglobin on pK′ to be of the same order of magnitude for ox and for human haemoglobin, and the few values they give for dog haemoglobin are similar. It would seem, therefore, that species differences in this respect are not large. It is of interest that the percentage of reduced haemoglobin in the perfusate in the tests accompanied by an increase in pH was usually high (mean 81·4 %), while it was unusually low (23–43 %) in three of the six tests unaccompanied by a change in pH.

SUMMARY

1. Ventilation of isolated perfused cat lungs with N_2 or 7 % CO_2 in N_2 instead of air or 7 % CO_2 in air causes a rise in pulmonary arterial pressure in preparations perfused at constant volume inflow.

2. The most usual effect of hypoxia was to produce a small increase of pH in the perfusate.

3. The pressor response did not appear to be dependent on changes in pH or lactate concentration of the perfusate.

The authors wish to thank Dr R. D. Stedeford and Mrs M. W. Duckworth, who assisted in some of the experiments. Mrs Marion Nixon gave invaluable technical help. Part of the apparatus was bought with a grant (to H.N.D.) from the Central Research Fund of London University.

REFERENCES

Barker, S. B. & Summerson, W. H. (1941). The calorimetric determination of lactic acid in biological material. *J. biol. Chem.* **138**, 535–554.

Daly, I. de B. (1938). Observations on the blood-perfused lungs of the dog, guinea-pig and *Macacus rhesus*, with special reference to 'spontaneous' lung movements. *Quart. J. exp. Physiol.* **28**, 357–403.

Dill, D. B., Daly, C. & Forbes, W. H. (1937). The pK′ of serum and red cells. *J. biol. Chem.* **117**, 569–579.

Duke, H. N. (1951). Pulmonary vasomotor responses of isolated perfused cat lungs to anoxia and hypercapnia. *Quart. J. exp. Physiol.* **36**, 75–88.

Duke, H. N. (1954). The site of action of anoxia on the pulmonary blood vessels of the cat. *J. Physiol.* **125**, 373–402.

Duke, H. N. (1957). Observations on the effects of hypoxia on the pulmonary vascular bed. *J. Physiol.* **135**, 45–51.

DUKE, H. N. & KILLICK, E. M. (1952). Pulmonary vasomotor responses of isolated perfused lungs to anoxia. *J. Physiol.* **117**, 303–316.

DUKE, H. N. & STEDEFORD, R. D. (1959). O_2 diffusion in lung of the anesthetized cat. *J. appl. Physiol.* **14**, 917–922.

GERMAN, B. & WYMAN, J. (1937). The titration curves of oxygenated and reduced haemoglobin. *J. biol. Chem.* **117**, 533–550.

HUNTER, F. T. (1951). *The Quantitation of Mixtures of Haemoglobin Derivatives by Photoelectric Spectrophotometry*, 1st ed. p. 63. Springfield, Illinois: Charles C. Thomas.

JOELS, N. & MACNAUGHTON, J. L. (1957). A micro-pH electrode system suitable for routine laboratory use. *J. Physiol.* **135**, 1–2*P*.

LILJESTRAND, G. (1958). Chemical control of the distribution of the pulmonary blood flow. *Acta physiol. scand.* **44**, 216–240.

LOGARAS, G. (1947). Further studies of the pulmonary arterial blood pressure. *Acta physiol. scand.* **14**, 120–135.

NISELL, O. (1948). Effects of oxygen and carbon dioxide on the circulation of isolated and perfused lungs of the cat. *Acta physiol. scand.* **16**, 121–127.

SCHOLANDER, P. F. & ROUGHTON, F. J. W. (1943). Micro gasometric estimation of blood gases. I. Oxygen. *J. biol. Chem.* **148**, 541–550.

VON EULER, U. S. & LILJESTRAND, G. (1946). Observations on the pulmonary arterial blood pressure in the cat. *Acta physiol. scand.* **12**, 301–320.

J. Physiol. (1960), **153**, *pp.* 423–432
Printed in Great Britain

THE STATE OF FACTOR I IN RAT BRAIN: THE EFFECTS OF METABOLIC CONDITIONS AND DRUGS

BY K. A. C. ELLIOTT AND N. M. VAN GELDER

From the Donner Laboratory of Experimental Neurochemistry, Montreal Neurological Institute, and the Department of Biochemistry, McGill University, Montreal, Canada

(*Received* 29 *February* 1960)

The term Factor I refers to the substance or substances in brain which inhibit impulse generation in the stretch receptor neurones of crustaceans (Florey, 1954). Gamma-aminobutyric acid (GABA) shows strong Factor I activity, can account for most of the activity in whole brain, and exerts marked effects on the physiological activity of brain and other organs (see Elliott, 1958; Elliott & Jasper, 1959).

In previous publications (Elliott & Florey, 1956; Elliott & van Gelder, 1958) it was shown that much of the Factor I in brain is present in a bound or occult form from which it is released by heat, suspension in hypotonic medium, or mild acid or alkali. In the present studies an attempt has been made to ascertain the significance of the difference between free and occult Factor I, to determine whether drug-induced changes in the GABA content of brain can be correlated with changes in Factor I content, and to determine the effects of drugs on the proportions of the free and occult forms of the Factor.

No definite conclusion could be drawn about the intracellular localization of occult Factor I, since this factor is released when brain tissue is suspended in media which are commonly used for the separation of subcellular particles. Decreases in the GABA content of brain have been found after administration of hydrazide (Killam & Bain, 1957) and insulin (Cravioto, Massieu & Izquiero, 1951), and increases after hydroxylamine administration (Baxter & Roberts, 1959). Substances other than free GABA are apparently present in brain and exert Factor I activity (McLennan, 1957, 1959) and effects of a variety of other inhibitory substances have been described (see Elliott & Jasper, 1959) but the present work shows that the changes in chemically-determined GABA content are paralleled by changes in Factor I activity. It is also shown that hypoxia and iproniazid cause changes in Factor I activity. The agents which decrease Factor I usually affect mainly the occult form and those which increase it affect mainly the free form. A number of neurotropic drugs have no obvious effect on the amount and condition of Factor I.

METHODS

Hooded rats weighing about 200 g were used. Drugs were administered intraperitoneally, unless otherwise specified, to rats which had been deprived of food since the previous day. The animals were decapitated and their cerebral hemispheres studied. For routine determinations of free and occult Factor I the tissue was immediately homogenized, by means of a Potter–Elvehjem homogenizer, in 4 volumes of potassium-free crayfish saline containing 5 mM glutamate, and the suspension was centrifuged at about 16,000 *g*. The Factor I activity found in the supernatant fluid is referred to as 'free Factor I' and the activity found in the residue, after resuspension and heating for 15 min, is referred to as 'occult Factor I'. Variations from this initial procedure are noted below and in Table 1.

The method for assaying Factor I and the solutions used for making and diluting tissue suspensions are those described previously (Elliott & Florey, 1956; Elliott & van Gelder, 1958) except as noted later. 'Crayfish saline' (CS; van Harreveld, 1936) contains (mM): NaCl 205, KCl 5·4, $MgCl_2$ 2·6, $CaCl_2$ 13·3. Potassium-free crayfish saline was the same but with KCl omitted. When necessary, small volumes of concentrated salt solutions were added after centrifuging to the supernantant fluids and residues to make their salt concentrations equal to that of crayfish saline. Resuspension of residues and further dilutions of all extracts for assay were made with complete tris-maleate buffered (0·01 M, pH 6·5) crayfish saline containing 5 mM glutamate. Factor I activities are expressed in terms of the amounts of GABA (μg) which would show the same activity, per gram of brain. None of the drugs used were found to affect the sensitivity of stretch receptor preparations to GABA, even in concentrations considerably higher than were likely to be present in the brain extracts as diluted for assay.

RESULTS

Free and occult Factor I in brain dispersed in various media

The results given in Table 1 show that when brain is homogenized in saline medium (modified crayfish saline) the proportion of the total Factor I which is found occluded in the centrifuged residue is rather constant, in spite of quite wide variations in the total Factor I activity found in the brains of individual animals. The volume of saline medium used in the initial homogenization and the length of time of homogenization had no obvious effect, nor did the use of mammalian Ringer-type solution or normal saline for the initial dispersion. Further, after grinding the tissue thoroughly with sand about the same amount of occult Factor I was found as after ordinary homogenization. In sand-ground tissue there was some increase in free and total Factor I but this might be accounted for by production by the tissue of extra, free Factor I during the time required for grinding. It was usually noted that elevated Factor I activity, particularly in the free condition, was found when whole brain or brain suspensions were allowed to stand at room temperature before heating or centrifuging (cf. Elliott & Florey, 1956).

When brain was homogenized in sucrose solutions of various concentrations, cold or at room temperature, the proportion of the total Factor I activity found in the free condition was considerably increased. The presence

of 0·1 mM ethylenediamine tetraacetate (Versene) or 7·5% polyvinylpyrrolidone (PVP) did not prevent this liberation of Factor I. The presence of low concentrations of sodium, magnesium or calcium chloride in sucrose solution decreased the liberation appreciably.

Brain hemispheres were homogenized in cold 0·25M sucrose + 0·1 mM Versene; the suspension, the pH of which was between 6·9 and 7·1, was subjected to differential centrifugation and the sediments were assayed

TABLE 1. Occult and free Factor I in rat brain dispersions

Initial homogenization medium, etc.*		Factor I activity, (μg γ-aminobutyric acid/g fresh brain) Occult	Free	Total	% free
CS + glutamate, no K† (averages ± S.D., 29 animals)		212 ± 27	108 ± 10	321 ± 31	34 ± 4
CS + glutamate, low K† (19 vol.)		186, 200, 213	130, 101, 123	316, 301, 336	41, 34, 37
CS + glutamate, low K†, 1–4° C		197	85	282	30
Sodium chloride (154 mM)		184	110	294	37
Mammalian Ringer-type solution‡		202, 223, 240	132, 123, 150	334, 346, 390	37, 36, 38
Ground with sand 15 min, then in CS + glutamate, no K		202, 256	160, 139	362, 395	45, 35
Stood, not ground, 15 min§	(Range of 7)	208 to 270	143 to 178	353 to 448	40 to 45
Sucrose 0·25 M	18,000 *g*	80, 83	201, 247	281, 330	71, 75
Sucrose 0·25 M, 1–4° C	18,000 *g*	91	211	302	70
Sucrose 0·25 M + Versene 0·1 mM, 1–4° C	18,000 *g*	58, 64	284, 217	342, 281	82, 77
Sucrose 0·25 M + PVP 7·5%, 1–4° C	18,000 *g*	84, 104	210, 169	294, 273	72, 62
Sucrose 0·3 M		65	220	285	77
Sucrose 0·3 M + NaCl 15 mM		136	255	391	65
Sucrose 0·3 M + $MgCl_2$ 2·6 mM		130	230	360	64
Sucrose 0·3 M + $MgCl_2$ 6·5 mM		140	220	360	61
Sucrose 0·3 M + $CaCl_2$ 3·0 mM		154	225	379	60
Sucrose 0·3 M + $CaCl_2$ 13·5 mM		165	170	335	51
Sucrose 0·4 M		88	174	262	66
Sucrose 0·4 M, 1–4° C	18,000 *g*	88	222	310	72
Sucrose 0·88 M, 1–4° C	18,000 *g*	31	268	299	90

* The suspensions were prepared in 4 volumes of medium at room temperature and centrifuged at about 16,000 *g* unless otherwise specified. † Crayfish saline containing 5 mM glutamate, K omitted or reduced in ititial homogenization to allow for K in tissue. ‡ Bicarbonate-buffered solution with composition of average spinal fluid. § The tissue was stood dry, or in medium, before homogenization, or homogenized and then stood; the results were about the same in all cases.

after being heated. The fraction 'nuclei and debris', which sedimented in 10 min at 1100 *g*, showed no more activity than the equivalent volume of supernatant fluid. The 'mitochondrial fraction', which sedimented in 15 min at 12,500 *g*, and the 'microsomal fraction' obtained after 30 min at 18,000 *g* each contained 30–40 μg/g brain more than could be accounted for by the content of the fluid remaining in these sediments. The final supernatant fluid contained 220–280 μg/g brain.

Factor I in brains of drug-treated animals

Results of determinations of free and occult Factor I in saline suspensions of brain from treated animals are shown in Table 2. Cravioto *et al.* (1951) found that the GABA content, determined by the chromatographic–colorimetric method, of the brains of rats is reduced after treatment of the animals with insulin. In agreement with this we find that the total

TABLE 2. Occult and free Factor I in brains from treated rats

Treatment (dose/kg*)	No. of animals	Factor I activity (μg γ-aminobutyric acid/g fresh tissue) Occult*	Free*	Total*	Symptoms immediately before decapitation†
None	29	212 ± 27 (S.D.)	108 ± 10 (S.D.)	321 ± 31 (S.D.)	None
Insulin 100–185 u.	2	195, 205	111, 136	309, 341	Beginning to be unsteady
	4	175 160–216	125 106–140	305 268–333	Coma up to 5 min, occasional convulsions
	2	137, 145	112, 114	249, 260	Coma for 10 min, occasional convulsions
	3	120 114–132	90 84–98	210 198–230	Coma for 25–30 min, occasional convulsions
	3	115 101–122	90 80–105	205 190–222	Coma for 60–180 min, occasional convulsions
Hypoxia	2	237, 266	180, 177	417, 443	Comatose; some movement possibly convulsive (30 min)
Anoxia	2	213, 217	120, 138	333, 355	Excited, then comatose. Died at 10 min
Thiosemicarbazide					
20 mg	2	171, 190	100, 98	290, 289	Convulsions for 20 min
30 mg	2	135, 148	110, 100	245, 248	Convulsions for 20 min
Semicarbazide					
30 mg	2	180, 190	113, 100	293, 290	None (90 min)
30 mg	2	210, 272	116, 117	326, 389	None (5 hr)
100 mg	1	191	103	294	None (90 min)
100 mg	1	172	94	266	Slight convulsions (90 min)
250 mg	2	125, 138	54, 44	179, 182	Convulsions for 10, 20 min
Hydroxylamine 50 mg	4	251 226–286	193 126–336	443 350–622	Twitching and cyanotic, then lethargic and cyanosis gone (90 min)
Metrazol 50 mg, 100 mg	2	226, 260	114, 131	340, 390	Convulsions for 6, 7 min
Megimide 16–25 mg	6	225 200–255	147 140–170	372 355–399	Convulsions for 10–85 min
Picrotoxin 9 mg	2	194, 218	133, 157	327, 375	Convulsions for 10, 20 min
Pentothal 100 mg	4	202 173–240	87 78–105	289 250–345	Anaesthetized for 10–15 min
Pentobarbital 50, 100 mg	2	230, 265	100, 105	330, 365	Anaesthetized for 10 min
Ether inhalation	2	204, 216	103, 106	307, 322	Anaesthetized for 10 min
Diphenyl hydantoin 50 mg intramusc.	2	196, 210	105, 110	301, 325	None (30 min)
Acetazoleamide 10 mg	2	197, 206	85, 93	290, 290	Sedated (120 min)
Reserpine					
5 mg	2	208, 266	119, 150	327, 416	Shivering, piloerection, lethargic (120 min)
2·5 mg × 5	4	220 210–240	125 124–135	345 (334–375)	
Chlorpromazine 5 mg	2	212, 219	105, 123	317, 342	None (120 min)
Iproniazid					
50 mg	2	193, 201	122, 147	315, 348	Quiet; no obvious symptoms (120 min)
50 or 100 mg × 2 to 5	12	225 196–226	165 120–240	390 341–450	
Iproniazid + Megimide 50 mg × 3 ipro. + 22 mg Meg.	1	245	210	455	Brief convulsion (60 min)
Iproniazid + reserpine					
50 mg ipro. + 5 mg × 2 res.	2	215, 221	167, 126	347, 381	As with reserpine alone (120 min)
50 mg × 3 ipro. + 5 mg res.	3	205 192–214	180 148–215	380 362–422	Excited, stereotyped behaviour (120 min)
Iproniazid + thiosemicarbazide	1	202	180	382	One brief convulsion, then normal (95 min)
100 mg × 2 ipro. + 20 mg thiosem.	1	173	117	290	Convulsions 25 min
50 mg × 2 + 100 mg ipro. + 20 mg thiosem.	1	203	171	374	Intermittent brief convulsions then normal (15 min)
	2	163, 175	131, 130	294, 304	Convulsions 15, 33 min

TABLE 2 (*cont.*)

Treatment dose/kg*	No. of animals	Occult*	Free*	Total*	Symptoms immediately before decapitation
Iproniazid + semicarbazide 100 mg × 2 ipro. + 250 semicarb.	2	118, 145	97, 101	215, 246	Convulsions 20, 30 min
Harmine HCl 10 mg	2	245, 264	126, 122	371, 386	Spastic movements then quiet and unsteady (180 min)
Harmaline HCl 10 mg	2	218, 240	153, 135	371, 375	Spastic, then quiet and unsteady (180 min)
SKF-trans 385 10 mg	2	242, 252	126, 119	368, 371	Very fearful, salivating (90 min)

* Where more than two results were obtained the averages and ranges are shown (except for the controls). Repeated doses of iproniazid were usually given, two/day (10 a.m. and 5 p.m.). The notation '× 3', for instance, indicates 3 doses. When other drugs were given with iproniazid these were given 10 min after the last dose of iproniazid. † Times given between brackets are the times between the last dose of a drug and decapitation of the animal.

Factor I content decreases markedly, reaching about 65 % of the normal value. The decrease is most apparent in the occult Factor I.

Hypoxia (animal in a desiccator filled with 5 % O_2 in N_2) for 30 min causes a considerable increase in total Factor I, mostly accounted for by increase in the free form. Under anoxic conditions (5 % CO_2 in N_2) there was less change in the levels of Factor I but the animals died in a few minutes.

Killam & Bain (1957) have shown that administration of carbazides to rats causes a decrease in the chemically-determined GABA content of the brain and this decrease is associated with the development of seizures. Results given in Table 2 show marked reductions in total Factor I activity after administration of thiosemicarbazide and particularly after semicarbazide in the large dose required to produce convulsions. With thiosemicarbazide the decrease in Factor I occurred in the occult fraction; with the large dose of semicarbazide both fractions were decreased.

Baxter & Roberts (1959) have reported that administration of hydroxylamine causes an increase in the GABA level in various parts of the brains of rats. Figures in Table 2 show that hydroxylamine administration causes a variable increase in Factor I levels in cerebral hemispheres, the effect being most marked in the free Factor I. In our experiments no convulsions were observed, though Baxter & Roberts, with higher doses, observed convulsions for 10 min or less after administration of the drug.

The convulsant agents Metrazol (pentylenetetrazole), Megimide (methylethyl glutarimide) and picrotoxin caused no definite change from the normal Factor I levels, though there was a trend to a rise in the free and total Factor I levels. The anaesthetic agents pentothal, pentobarbital and ether caused no consistent changes in Factor I levels except that somewhat low levels occurred in two animals that received pentothal. Sodium diphenylhydantoin (Dilantin), acetazoleamide (Diamox) and chlorpromazine in the doses used had caused no obvious or consistent changes in Factor I

levels at the time when the animals were killed. Administration of reserpine, which is known to release bound serotonin, seemed to cause a slight elevation of free Factor I.

After repeated doses of iproniazid (Marsalid) the total Factor I was always moderately high; most of this increase was due to an increase in the level of free Factor I. The averages for total and free Factor I were highly significantly different from normal ($P < 0{\cdot}01$).

High values for free and total Factor I were obtained also when iproniazid was administered before Megimide or reserpine. When iproniazid was administered before thiosemicarbazide or semicarbazide there seemed to be the tendency of the hydrazides to lower the Factor I content and the tendency of iproniazid to raise the free Factor I. As a consequence, when both thiosemicarbazide and iproniazid had been administered the total Factor I content was at about the normal level. In spite of this the animals suffered convulsions. Though the total Factor I was not below normal in these cases, the occult Factor I *was* usually below normal, but was accompanied by a rather high amount of the free factor.

Three other monoamine oxidase inhibitors, harmaline and harmine (Sjoerdsma, Gillespie & Udenfriend, 1959; Tabachnik & Rubin, 1959) and tranylcypromine (SKF-trans 385; Tedeschi, Tedeschi, Ames, Cook, Mattis & Fellows, 1959) were tested in single doses (10 mg/kg). These tended to raise the free, occult and total Factor I levels.

DISCUSSION

The amount of occult Factor I found in brains from untreated animals was not obviously affected by varying the length of time of homogenization or the volume of saline medium, or even by grinding the tissue with sand. This seems to indicate that the free and occult fractions represent different fractions which actually exist in brain; the division into these two fractions does not seem to be an artifact resulting from the mechanical disruption of the tissue. Further evidence for this is the fact that incubation of brain suspensions with added GABA does not produce an increase in occult Factor I, whereas with brain slices such an increase does occur (Elliott & van Gelder, 1958). It is probable, however, that the free Factor I fraction found does not represent truly free, extracellular Factor I present in brain *in vivo*. This is evident from the fact that, after incubating slices of cerebral cortex aerobically in saline medium, no detectable Factor I is found in the medium, even though the slices continue to contain about 100 μg of 'free' Factor I per gram (plus about 200 μg of occult Factor I per gram; Elliott & van Gelder, 1958). Further, electron microscope studies by a number of authors indicate that there is virtually no extracellular space in the central nervous system. Free Factor I can apparently

diffuse out of brain to a slight extent, since Florey & McLennan (1955) detected Factor I in fluids which had been in contact with the brains of living cats and traces of GABA have been detected in human cerebrospinal fluid (Knauff, 1958; Logothetis, 1958).

In attempts to localize the occult Factor I in subcellular particles, by suspension in sucrose solution and differential centrifugation, it was found that salt-free sugar solutions caused release of Factor I from its occult form. The mechanism by which Factor I is occluded is apparently different from those that apply to acetylcholine, adrenaline, noradrenaline and serotonin, since all these remain to a large extent in the bound forms during separation of subcellular particles in sucrose media (e.g. Hebb & Whittaker, 1958; Eade, 1958; Walaszek & Abood, 1959).

Dawson (1950, 1953) and Cravioto *et al.* (1951) have shown that the glutamate content of brain decreases during insulin hypoglycaemia. This decrease in the level of the substance from which GABA is formed would explain adequately the fall in Factor I level following insulin administration. The increase in the levels of Factor I, especially of the free Factor, during hypoxia might be due to decrease in the level of α-ketoglutarate in the brain with consequent decrease in the rate of removal of GABA by transamination.

As has been clearly indicated by the work of Killam & Bain (1957) and Roberts, Rothstein & Baxter (1958) on the metabolism of GABA, the fall in Factor I level after carbazide administration can be accounted for by inhibition of glutamate decarboxylase through combination of the carbazide with the co-enzyme pyridoxal phosphate. Baxter & Roberts (1959) pointed out that hydroxylamine is a more effective inhibitor of GABA-α-ketoglutarate transaminase than of glutamate decarboxylase, and this might account for the elevation of brain GABA after hydroxylamine administration. The trend toward a rise in Factor I after administration of Metrazol, Megimide and picrotoxin might be connected with hypoxic states produced by these convulsants.

As far as we are aware the possibility that GABA can serve as a substrate for monoamine oxidase has not been tested. The increase in free Factor I in the brain following repeated doses of iproniazid suggests that monoamine oxidase may be to some extent involved in the metabolism of GABA. Another possibility is that accumulation of some other amine may interfere with the metabolism of Factor I.

Interference with production of Factor I, as in insulin hypoglycaemia and hydrazide intoxication, results predominantly in a decrease in the amount of the occult form. Interference with further metabolism, as perhaps during hypoxia or after iproniazid or hydroxylamine treatment, results predominantly in an increase of the free form. These observations

suggest that Factor I is initially produced in the occult form and that it is mainly the free Factor I, released from the occult form, which undergoes further metabolism.

When thiosemicarbazide and iproniazid are present together, the level of occult Factor I in the brain tends to fall and free Factor I tends to accumulate and the total Factor I content can be in the normal range though convulsions still occur. The evidence provided by Killam & Bain (1957) and Killam (1957), that the convulsive effects of the hydrazides result from their effect on the GABA content of the brain, is strong. From the present results with the hydrazides alone and with iproniazid it seems that it is mainly the level of the occult fraction of the Factor I which is concerned in convulsive effects. Insulin hypoglycaemia is accompanied also by a decrease in the occult form and there may be a relation between this decrease and the convulsions that occur. There is no doubt that solutions of free GABA applied directly to the brains of animals *in vivo* exert inhibitory actions and oppose the action of the hydrazides (Purpura, Girado & Grundfest, 1957; Iwama & Jasper, 1957; Killam & Killam, 1958; Dasgupta, Killam & Killam, 1958). It is, however, reasonable to suppose that much of this GABA is absorbed into the occult condition by the tissue, just as GABA is absorbed by brain slices (Elliott & van Gelder, 1958), so that the level of occult GABA is raised. On this hypothesis we would not expect the elevation of free GABA, as in hypoxia or after hydroxylamine administration, to affect the susceptibility of animals to convulsions. Eidelberg, Baxter, Roberts, Saldias & French (1959), however, have produced evidence that the raised levels of GABA produced in cerebral cortex by hydroxylamine administration are associated with raised electrographic seizure thresholds. Either the increase in free Factor I *is* effective or the associated smaller increase in occult Factor I is the significant factor.

No consistent effects on free or occult Factor I were found to be associated with most of the other drugs tested. It seems that the main effects of these drugs are not mediated through changes in Factor I unless such changes occur only in restricted specific regions of the brain.

SUMMARY

1. The proportion of 'free' (extractable with saline medium without heat) to 'occult' (liberated by heat) Factor I in normal rat brains is fairly constant. The occult Factor I is not released by grinding the tissue with sand.

2. When brain tissue is suspended in salt-free sucrose solutions most of the occult Factor I becomes free.

3. The total Factor I content is decreased in brains from rats suffering from coma and convulsions after insulin treatment and from rats suffering from convulsions induced by hydrazides. The decrease after insulin and thiosemicarbazide is mainly in the occult fraction.

4. The total Factor I content is increased by hypoxia, hydroxylamine administration and by repeated doses of iproniazid. These increases are most marked in the free fraction.

5. No obvious changes in Factor I content were observed after administration of various other convulsant, anaesthetic or psychotropic drugs.

This work was aided by grants from the Banting Research Foundation and the Wellcome Trust.

REFERENCES

Baxter, C. F. & Roberts, E. (1959). Elevation of γ-aminobutyric acid in rat brain with hydroxylamine. *Proc. Soc. exp. Biol., N.Y.*, **101**, 811–815.

Cravioto, R. O., Massieu, G. & Izquiero, J. J. (1951). Free amino-acids in rat brain during insulin shock. *Proc. Soc. exp. Biol., N.Y.*, **78**, 856–863.

Dawson, R. M. S. (1950). Studies on the glutamine and glutamic acid content of the rat brain during insulin hypoglycaemia. *Biochem. J.* **47**, 386–391.

Dawson, R. M. C. (1953). Cerebral amino acids in fluoroacetate-poisoned, anaesthetized and hypoglycaemic rats. *Biochim. biophys. acta*, **11**, 548–552.

Dasgupta, S. R., Killam, E. K. & Killam, K. F. (1958). Drug action on rhinencephalic seizure activity in the cat. *J. Pharmacol.* **122**, 16*A*.

Eade, N. R. (1958). The storage and release of catechol amines. *Rev. canad. Biol.* **17**, 299–311.

Eidelberg, E., Baxter, C. F., Roberts, E., Saldias, C. A. & French, J. D. (1959). Anticonvulsant properties of hydroxylamine and elevation of cerebral γ-aminobutyric acid in cats. *Proc. Soc. exp. Biol., N.Y.*, **101**, 815–817.

Elliott, K. A. C. (1958). γ-Aminobutyric acid and factor I. *Rev. canad. Biol.* **17**, 367–388.

Elliott, K. A. C. & Florey, E. (1956). Factor I—inhibitory factor from brain. Assay. Condition in brain. Simulating and antagonizing substances. *J. Neurochem.* **1**, 181–191.

Elliott, K. A. C. & Jasper, H. H. (1959). γ-Aminobutyric acid. *Physiol. Rev.* **39**, 383–406.

Elliott, K. A. C. & van Gelder, N. M. (1958). Occlusion and metabolism of γ-aminobutyric acid by brain tissue. *J. Neurochem.* **3**, 28–40.

Florey, E. (1954). An inhibitory and an excitatory factor of mammalian brain and their action on a single sensory neuron. *Arch. int. Physiol.* **62**, 33–53.

Florey, E. & McLennan, H. (1955). The release of an inhibitory substance from mammalian brain and its action on peripheral synaptic transmission. *J. Physiol.* **129**, 384–392.

Hebb, C. C. & Whittaker, V. P. (1958). Intracellular distribution of acetylcholine and choline acetylase. *J. Physiol.* **142**, 187–196.

Iwama, K. & Jasper, H. H. (1957). The action of gamma-aminobutyric acid upon cortical electrical activity in the cat. *J. Physiol.* **138**, 365–380.

Killam, E. K. & Killam, K. F. (1958). Pharmacological studies of evoked cortical potentials. *J. Pharmacol.* **122**, 37*A*.

Killam, K. F. (1957). Convulsant hydrazides. 2: comparison of electrical changes and enzyme inhibition induced by the administration of thiosemicarbazide. *J. Pharmacol.* **119**, 263–271.

Killam, K. F. & Bain, J. A. (1957). Convulsant hydrazides. 1: In vitro and in vivo inhibition of vitamin B_6 enzymes by convulsant hydrazides. *J. Pharmacol.* **119**, 255–262.

Knauff, H. G. (1958). The free amino-acids of the cerebrospinal fluid. *Nature, Lond.*, **182**, 937.

LOGOTHETIS, J. (1958). Free amino acid content of cerebrospinal fluid in humans and dogs. *Neurology*, **8**, 299–302.

MCLENNAN, H. (1957). A comparison of some physiological properties of an inhibitory factor from brain (Factor I) and of γ-aminobutyric acid and related compounds. *J. Physiol.* **139**, 79–86.

MCLENNAN, H. (1959). The identification of one active component from brain extracts containing Factor I. *J. Physiol.* **146**, 358–368.

PURPURA, D. P., GIRADO, M. & GRUNDFEST, H. (1957). Selective blockade of excitatory synapses in the cat brain by γ-aminobutyric acid. *Science*, **125**, 1200–1202.

ROBERTS, E., ROTHSTEIN, M. & BAXTER, C. F. (1958). Some metabolic studies of γ-aminobutyric acid. *Proc. Soc. exp. Biol., N.Y.*, **97**, 796–802.

SJOERDSMA, A., GILLESPIE, L. & UDENFRIEND, S. (1959). A method for measurement of monoamine oxidase inhibition in man: application to studies on hypertension and its amine oxidase inhibitors. *Ann. N.Y. Acad. Sci.* **80**, 969–980.

TABACHNIK, I. I. A. & RUBIN, A. A. (1959). Some relationships between peripheral monoamine oxidase inhibition and brain 5-hydroxytryptamine levels in rats. *Proc. Soc. exp. Biol., N.Y.*, **101**, 435–437.

TEDESCHI, R. E., TEDESCHI, D. H., AMES, P. L., COOK, L., MATTIS, P. A. & FELLOWS, E. J. (1959). Some pharmacological observations on tranylcypromine (SKF trans-385); a potent inhibitor of monoamine oxidase (25256). *Proc. Soc. exp. Biol., N.Y.*, **102**, 380–381.

VAN HARREVELD, A. (1936). A physiological salt solution for freshwater crustaceans. *Proc. Soc. exp. Biol., N.Y.*, **34**, 428–432.

WALASZEK, E. & ABOOD, L. G. (1959). Fixation of 5-hydroxytryptamine by brain mitochondria. *Proc. Soc. exp. Biol., N.Y.*, **101**, 37–40.

J. Physiol. (1960), **153**, *pp.* 433–446
With 2 plates and 2 text-figures
Printed in Great Britain

PREFERRED SITES OF ADENINE NUCLEOTIDE IN FROG'S STRIATED MUSCLE

By D. K. HILL

From the Postgraduate Medical School of London, Hammersmith Hospital, London, W. 12

(*Received* 6 *April* 1960)

The location of tritium-labelled adenine nucleotide in frog's striated muscle has been investigated by autoradiography in freeze-dried material (Hill, 1959) and after fixation with osmium tetroxide and lanthanum (Hill, 1960). After freeze-drying there was only a small degree of preferential siting of nucleotide; about 20% of the total appeared to lie in a narrow band near the boundary between the A and I bands. The location at a particular site was much more marked in a muscle which had been fixed in a mixture of osmium tetroxide and lanthanum; it was shown that some 70% of the adenine nucleotide was located in the region of the N bands (in the isotropic part of the sarcomere).

The ultimate objective is, of course, to determine where the adenine nucleotide is located in *living* muscle, but it is very difficult to achieve this because the fixation process is liable to result in a disturbance of the balance of forces responsible for holding this mobile and unstable material at some particular position within the sarcomere. Now, after the experiments to be described, the question as to the location in normal muscle is still not answered; indeed the situation is more complicated than it had previously appeared to be. One of the methods of fixation which has been used for the experiments described here is possibly less subject than the others have been to the objection that it disturbs the siting of the nucleotide. It involves rapid freezing of a thin muscle (m. ext. long. dig. IV), followed by freeze-substitution in a solution of osmium tetroxide and lanthanum nitrate in ethanol. This has resulted in the nucleotide becoming sited largely in the region of the so-called 'A lines' (Hill, 1960) which lie inside the A band, close to the boundary with I.

Though the meaning, in physiological terms, is obscure, it is interesting to know of the existence of at least two preferred sites for nucleotide in the sarcomere. The emphasis in this paper is on the demonstration, and on the fact of the existence, of preferred sites, for little can be said concerning what variations in the conditions of a normal muscle are able to cause one or other of these potential centres of aggregation to become involved.

Another unexpected result has come out of the present series of experiments. Acid-soluble nucleotide in muscle is, in part, bound against extraction by water (see Hill, 1959). An experiment was made with the purpose of locating the bound fraction by fixing a muscle in aqueous osmium tetroxide without the addition of lanthanum nitrate. The fixative was not capable of precipitating nucleotide and it was assumed that, during fixation, the free nucleotide would escape into the solution leaving the bound fraction to be located. This is not, however, what actually happened, for none of the nucleotide escaped from a muscle fixed in this way. What is more, the nucleotide assumed a different siting pattern from that seen when lanthanum was present in the fixative, the A-line region now being the one preferred.

METHODS

Preparation of tritiated adenine. A fresh batch of tritiated adenine was prepared by essentially the same method as that used before (Hill, 1959). On this occasion a higher specific activity was procured by using a smaller reaction volume. In the previous method acetic acid was used as the solvent, and the reaction volume could not be reduced because of the low solubility of adenine in acetic acid. It was found that adenine is ten times more soluble in formic acid than in acetic acid at 100° C, so in this solvent a considerably higher specific activity should be produced with a given amount of adenine and of tritium.

The reaction mixture consisted of 22·9 mg adenine, 1·0 mg Adam's platinum catalyst (PtO_2) and 54·1 μl. (65·5 mg) 90 % formic acid; these substances were introduced down a long funnel into the curved bottom of a Pyrex glass tube, of internal diameter 7 mm, length 20 cm. A tap was fitted to the top of the tube. At 20° C adenine at this concentration does not dissolve, but at 100° C it readily does so, though it forms a semi-solid mass on re-cooling. The reaction mixture was frozen in liquid oxygen, the vessel was evacuated, then filled with hydrogen and warmed to 100° C. The reaction mixture was then gently swirled around the bottom of the tube for a few minutes. The freezing, evacuation, filling with hydrogen and mixing were repeated twice, and finally the reaction mixture was spread in a thin semi-solid layer by rotating the tube while cooling to room temperature. The reaction vessel was then left at room temperature for 23 hr to ensure complete reduction of the catalyst. The tritium gas (5 curies, 97·1 % tritium, 2·04 ml. at s.t.p.) was converted to tritium oxide which was distilled over into the reaction vessel. The latter was sealed off in a flame. The volume of the sealed vessel was 7 ml. (This volume has to be taken into account because of the need to limit the amount of solvent in the vapour phase to a small proportion of the whole. It was calculated that in this case the proportion was 15 %.) The reaction tube was then set up in an oven at 100° C and the mixture kept in motion for 112 hr by continuous rotation of the tube, in an almost horizontal position, at about 20 revolutions/min, by means of an electric motor. The solvent was then removed by distillation on a vacuum line and collected in a receiver cooled in liquid oxygen. The labile tritium atoms of the adenine were then exchanged for ordinary hydrogen, and the product separated from the platinum by the following procedure. The adenine was dissolved in 5 ml. N-NH_4OH; 20 mg $MgSO_4 . 7H_2O$ was added to flocculate the colloidal platinum. The mixture was centrifuged. The clear solution was separated and the solvent removed by evaporation. The adenine was redissolved in 10 ml. N formic acid. After standing for about 30 min the solvent was removed by evaporation.

The product was assayed spectrophotometrically and, on a scintillation counter, for its specific activity. The yield was 20·3 mg adenine, and the specific activity was 1·50 c/m-mole;

this is 4·5 times the activity obtained by the previous method. The theoretical maximum after complete equilibration of the tritium with all the hydrogen atoms in the reaction mixture would be 2·50 c/m-mole. If it is assumed that there is equal probability of labelling in the two stable positions of the adenine molecule, the present activity of 1·50 c/m-mole means that a fraction 1 in 21 of the molecules is labelled with one tritium atom, and 1 in 440 is labelled in both stable positions. The half-life of tritium is 12·5 yr, and all activities quoted are corrected for loss by decay.

Labelling the muscles. The muscles were labelled as before (Hill, 1960) by intraperitoneal injection of the frog with an aqueous solution of the tritiated adenine.

Frog 1. Weight 15 g. 12·2 mc ^{3}H-adenine injected in 1·1 ml. water (810 mc/g).

Frog 2. Weight 11 g. 12·5 mc ^{3}H-adenine injected in 1·15 ml. water (1140 mc/g).

The frogs were kept in a moist atmosphere at 20° C for 24 hr (frog 1), and at 15° C for 22 hr (frog 2) before dissection of the muscles.

Fixation of m. ext. long. dig. IV by freeze-substitution. The preservation of a muscle by freeze-drying at −40° C (Hill, 1959) was not satisfactory, even with a muscle as thin as the m. ext. long. dig. IV. It seems improbable that this could have been due to ice crystal formation during the rapid freezing at −160° C, and the more likely explanation is that ice crystal growth occurred later while the muscle was being dried at −40° C. The rate of ice crystal growth decreases rapidly as the temperature is lowered, but owing to the sharp fall in the vapour pressure of ice it is not practicable to dry a piece of tissue by vacuum sublimation at a temperature much below −40° C. The alternative method, known as freeze-substitution, in which the ice is replaced by ethanol (or other solvent which does not freeze) at a temperature of −80° C or below, can be used. Feder & Sidman (1958) have proposed that tissue fixatives may with advantage be dissolved in the substitution solvent, and these authors produce evidence to show that fixation of protein components can take place in such non-aqueous media at a low temperature. This method has been used here: in addition to adding osmium tetroxide to the ethanol, lanthanum nitrate was, at the suggestion of Dr Feder, also dissolved in the mixture. Whether lanthanum can combine with nucleotide, and thus immobilize it, in non-aqueous solution is doubtful, and there is no evidence either that it does in fact do so, or that the nucleotide does not (as a consequence of failure in this respect) redistribute itself during the prolonged substitution process. However, although there may be doubt as to the efficacy of this method in preserving the siting of the nucleotide, it does result in excellent preservation of the structure as seen in the light microscope, and the artifacts found in a freeze-dried muscle (Hill, 1959) are absent.

Frog 1 was used for this experiment. A toe muscle (m. ext. long. dig. IV) was dissected; this was mounted on a glass holder by cotton attached to the tendons, and it was then soaked for 90 min in oxygenated Ringer's solution (composition (mM): NaCl, 96; KCl, 5·0; $CaCl_2$, 4·0) at 0° C. The muscle was used in the normal polarized condition and to prevent the shock of immersion in the freezing fluid from setting up propagated impulses which might activate the unfrozen part this muscle was soaked for 10 min at 0° C in choline-substituted Ringer's solution (composition (mM): choline chloride, 106; KCl, 5·0; $CaCl_2$, 4·0). The muscle was stretched and then plunged into Arcton 6 (dichloro-difluoro-methane) cooled to −160° C in liquid oxygen. It was then rapidly transferred to the following mixture cooled to −75° C in acetone and solid carbon dioxide: 20 ml. ethanol; 0·2 g osmium tetroxide; 0·24 g La $(NO_3)_3$ $6H_2O$. (This mixture has to be prepared at a temperature not above 0° C. At 20° C the osmium tetroxide is rapidly reduced by the ethanol and the solution becomes black.) After 6 days in this solution at −75° C the muscle was rapidly transferred to aqueous fixative at 20° C (composition (mM): osmium tetroxide, 40 (1 %); TRIS (tris-(hydroxymethyl)-amino methane), 39; HNO_3, 31 (pH 7·4); La $(NO_3)_3$ $6H_2O$, 28) for 35 min. The subsequent procedure, involving removal of the fixative and dehydration of the muscle, was essentially the same as that described earlier (Hill, 1960).

Fixation of a sartorius muscle at room temperature in lanthanum-free solution. Both

sartorius muscles of frog 2 were dissected and fixed on supports. They were soaked for 60 min in oxygenated Ringer's solution at 0° C. Muscle 1 was treated before fixation by a procedure similar to that used for preparing the 'normal' muscle in the earlier experiments (Hill, 1960; muscle 1, frog 1); that is, it was given a preliminary soaking in oxygenated Ca-free mixture at 0° C for 75 min. It was next immersed for 10 min in oxygenated isotonic K_2SO_4 at 20° C. It was then fully stretched. The muscle was fixed, for 90 min, in a solution in which NaCl substituted, in equi-osmolar quantity, the lanthanum nitrate used previously (Hill, 1960), composition (mM): osmium tetroxide, 40 (1 %); TRIS, 39; HNO_3, 31 (pH 7·4); NaCl, 55; sucrose, 110. The subsequent procedure, involving removal of the fixative and dehydration of the muscle, was essentially the same as that described earlier (Hill, 1960). (Lanthanum was present in the solution used for washing out the fixative, and was introduced for the purpose of immobilizing such nucleotide as remained at this stage.)

The second muscle (muscle 2) was used as a control. It was made inexcitable by immersion for 5 min in oxygenated isotonic K_2SO_4 at 0° C, and it was then fixed in a mixture containing lanthanum (composition: see Hill, 1960), and prepared for embedding as before.

Portions of both muscles were retained after dehydration in ethanol for spectrophotometric analysis of their nucleotide contents.

Embedding, sectioning, autoradiography, staining. The procedure was essentially similar to that used earlier (Hill, 1960). As before, crystal violet was found to be the most suitable dye for staining the muscle, both in uncovered sections and in sections covered with the autoradiographic film. In some cases the sections were stained, as before, with 20 mM crystal violet dissolved in 100 mM acetate buffer at pH 4·0; in others 100 mM phosphate buffer at pH 7·5 was used.

The autoradiographs were examined with a high-power oil-immersion objective (N.A. 1·3), and photographed on Kodak P. 200 plates, with a colour filter to provide the required contrast. The relationship between the stained regions and the A and I bands was established, whenever necessary, by using polarized light.

RESULTS

The stain pattern with crystal violet

M. ext. long. dig. IV, fixed by freeze-substitution. The muscle showed clear N bands (Pl. 1, fig. 1) when the sections were stained with crystal violet at pH 4. (It should be pointed out that the N bands of muscle which have been described by earlier workers are *narrow* lines within the I band. The basophilic material referred to here occupies a region which may be relatively broad when the sarcomere is extended, and indeed the bands (Pl. 1, fig. 1) seem to occupy the whole of the I region except for the part containing the Z line. It is therefore perhaps misleading to refer to them as N bands; the phrase 'basophilic material in the N-band region' would not prejudge the issue, but it is avoided for the sake of brevity.) As in the earlier work (Hill, 1960) the pattern which was obtained depended upon the permeability of the methacrylate. The permeability could be reduced by heating the sections to 180° C for a few minutes before staining at room temperature. On the other hand, it was increased by raising the temperature slightly; for instance, if the stain solution was warmed to 40° C the muscle tissue in a section picked up the dye more readily than at room temperature. The pH of the solution also affected the permeability;

when the solution was at pH 7–8 the dye entered much more freely than it did at pH 4. An alkaline solution (for instance, photographic developer) caused an irreversible increase in the permeability of a section, so that even after subsequent washing the permeability remained abnormally high.

When the methacrylate was relatively impermeable the stain pattern, in this case simply N bands, was sharp and clear. The changes which occurred when the permeability was progressively increased were similar to those which have been described earlier (Hill, 1960); the first noticeable change was a blurring of the N bands, followed by an apparent 'splitting' of each band to give new lines of stain at the position of the Z lines and of the A lines. Finally, the stain was acquired by the greater part of the A band, so that the region between the A lines filled up and the latter were not separately discernible. When the stain was buffered at pH 7·5 only the 'final' stage in the transformation (i.e. a Z- and A-band pattern) was obtained (Pl. 2, fig. 2); under these conditions neither the N-band pattern nor the A lines were seen.

With the m. ext. long. dig. IV fixed by freeze-substitution the N-band pattern, seen only under conditions of high permeability and low pH, appeared to be unusually labile compared with that obtained previously (Hill, 1960). The transformation to the Z–A pattern occurred so readily that it was possible to be certain of obtaining a sharp, uniform, picture only if the stain was buffered at alkaline pH (7·5), in which case the Z–A pattern was fully brought out. For clarity in analysis the autoradiographs were stained in this way.

The spacing of the N bands has been referred to previously (Hill, 1960) and the dependence of the separation of the members of a pair upon the over-all sarcomere length was considered to be a matter of some interest, because of its possible bearing on the problem of deciding what structures possess, or contain, the basophilic material in question. There was some indication (Hill, 1960) that the spacing of the members of an N pair increased and decreased with the sarcomere length. Some further information on this point is now available. Sections from various parts of m. ext. long. dig. IV, fixed by freeze-substitution, showed a range of sarcomere lengths, and the corresponding spacings were measured. At sarcomere lengths of 2·9, 3·1, 3·3 and 4·0 μ the N-band spacings were respectively 0·88, 0·97, 1·0 and 1·2 μ. The figures quoted previously (Hill, 1960) were for sarcomere lengths of 2·7 and 3·1 μ, when the N-band spacings were respectively 0·74 and 0·98 μ. There seems little doubt that in this range of sarcomere lengths (2·7–4·0 μ) the N-band spacing increases and decreases with the length of the muscle. At shorter lengths no reliable figures are available to show whether the spacing continues to decrease as the muscle shortens further.

Sartorius muscle fixed in lanthanum-free solution. The stain pattern using crystal violet at pH 4 is shown in Pl. 1, figs. 2, 3. Even after taking the most stringent precautions to ensure low permeability of the methacrylate, the N bands were completely absent, and no intermediate stages in the transformation from the N-band to the Z–A-line pattern were ever seen. The pattern was mostly the one shown in Pl. 1, fig. 2, and consisted of Z and A lines with very little stain between them; a few fibres were stained more deeply, and in these (Pl. 1, fig. 3) the A band, between the A lines, had also taken up the stain.

The A lines lie just inside the A band, close to the boundary with the I band. There is some evidence, for sarcomeres of 2·2–2·7 μ, that the A lines retain this close relationship with the boundary as the length of the muscle changes, for their separation as measured across the A band was constant (Pl. 1, fig. 2), and it may be assumed that the A band itself has a fixed length over this range. It seems, therefore, that the stainable material in these A lines is not associated with the I filaments, for the latter are believed to move in and out of the A band when the length of the sarcomere changes. It follows that the A-line substance must be either extrafibrillar or, alternatively, 'tied' to the A filaments.

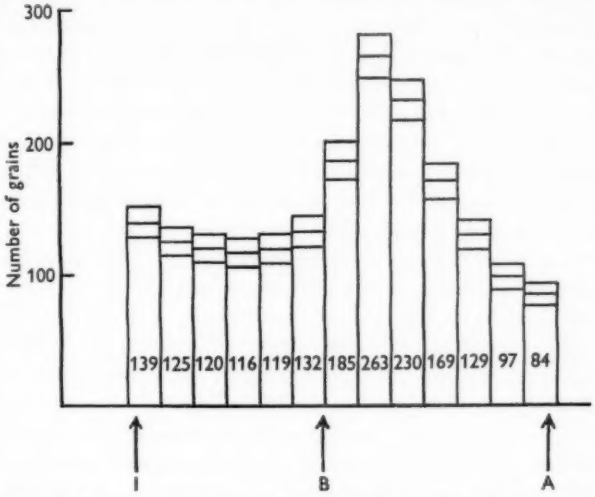
Autoradiography

M. ext. long. dig. IV, fixed by freeze-substitution. Autoradiographs of transverse and longitudinal sections were made. In transverse sections the distribution of radioactivity was found to be uniform, and differed in this respect from the non-uniform distribution seen previously in a muscle fixed at 20° C in osmium tetroxide and lanthanum. This difference was to be expected, since the factors presumed (Hill, 1960) to be responsible for the aggregation near the fibre surfaces in the unfrozen muscle would not be operative when a frozen muscle was fixed, for in the latter the lanthanum would penetrate to all parts of the fibre while the nucleotide was in an immobilized state.

Grain counts were made with autoradiographs of both transverse and longitudinal sections. From these counts an estimate was made (the basis of the calculation is given by Hill, 1959) of the isotope content of the muscle, expressed in terms of the amount of isotope injected per gram of frog. This varied from 0·1 to 0·3 μc/g muscle per μc/g frog, in different parts of the muscle. It is comparable with values found on other occasions.

The autoradiographs made with longitudinal sections were stained with crystal violet at pH 7·5. The distribution of the silver grains, in relation to the boundaries of the A and I bands, was measured in the same way as before (Hill, 1959, 1960). The result is shown in Text-fig. 1. There is clearly a peak in the distribution in the A band, at or about the A lines, while at

the centre of A there is a minimum in the frequency of occurrence. The distance between these two points is about 0·6 μ. According to an earlier calculation (Hill, 1959) a disk source, with its plane at right angles to the long axis of the muscle, would produce a yield of about 10–20 % of the maximum at a distance of 0·6 μ along the axis on either side of the disk. If it is as little as 10 % (and, in point of fact, the indications are (Hill, 1960) that the resolution is perhaps rather better than what had been calculated),



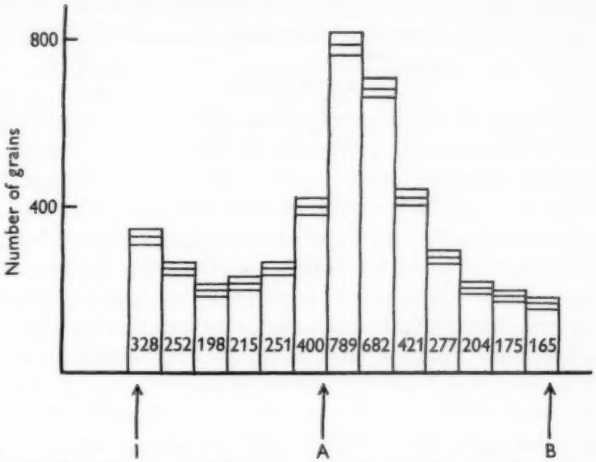
Text-fig. 1. Distribution of grains in autoradiographs of m. ext. long. dig. IV fixed by freeze-substitution. A total of 1908 grains is represented. These are grouped in thirteen blocks between the centre of the I band (arrow at left) and the centre of A (arrow at right). The arrow at B is placed 0·75 μ from A and represents the position of the A-I boundary. The variation expected from random fluctuations in the radioactive emission is indicated by the uppermost and the lowest horizontal lines at the top of each block; these are displaced by 1 S.D. above and below the recorded value. Sarcomere length, 2·74 μ. Muscle section 0·3 μ thick; exposure 11 days.

it follows that some of the nucleotide must be located in other regions of the A band besides that indicated by the peak in the distribution, for the frequency at the centre of A is 32 % of that at the peak. Suppose that there is a uniform level of activity in A in addition to the aggregate at the peak position. Roughly, this uniform 'background' will account for 64 grains in each of the seven blocks to the right of the diagram. It may then be calculated that the aggregate of nucleotide at the position of the peak amounts to 51 % of the whole. There is, in addition, a further source of radiation in the I band, and though there is some indication that this is in

the form of an aggregate on the Z line the standard errors in the counts are too large for the tendency in the distribution to have much significance.

Sartorius muscle fixed in a lanthanum-free solution. Before describing the results obtained by autoradiography it is necessary to digress for a moment. It had been expected that the absence of lanthanum from the fixative would result in a loss of nucleotide. The simplest and most accurate way of measuring the proportion actually lost was by making a spectrophotometric assay of the adenine nucleotide in a weighed portion of the fixed muscle, and then to compare this with the amount present in the control muscle which had been fixed with a lanthanum mixture. The two samples for analysis were taken after dehydration of the fixed muscles. They were dried and weighed. The nucleotide was extracted with 5% perchloric acid at 20° C. The extraction was found, by following the ultraviolet absorption, to occur rather slowly, being only about 70% complete in 90 min, and the extraction was continued for a total of 29 hr before the final measurement was made. The absorption spectra of the extracts showed that in both muscles some deamination of the adenine nucleotide had occurred, for the peaks were at 255 mμ instead of 260 mμ. The quantities were estimated by using a molecular extinction coefficient of $1{\cdot}3 \times 10^4$, which is intermediate between the value appropriate for adenylic compounds and that for the inosinic analogues. The fresh weights of the muscle samples were assumed to be five times the dried weights. The amount of nucleotide in muscle 1 (fixed in lanthanum-free solution) was 7·3 m-mole/kg; in muscle 2 (fixed in the presence of lanthanum) it was 6·0 m-mole/kg. These are in the range expected for normal muscle. It was assumed that nucleic acid was not extracted from the muscle, but in any case its contribution to the total optical density would be not more than 10% (unpublished observations). The accuracy in this estimation was largely determined by the accuracy of weighing the dried pieces of muscle. The weights were recorded as 2·4 and 2·9 mg, and these could have been in error by as much as 0·2 mg. It is therefore unlikely that there is any significance in the difference between the values estimated for the nucleotide contents in the two muscles. However, it is reasonably certain that most, if not all, of the nucleotide was retained in the muscle fixed in a lanthanum-free solution. This conclusion was confirmed by the subsequent estimate of the radioactivity of the muscle made from grain counts on the autoradiographs; this was 0·17 μc/g muscle per μc/g frog. It is supported also by the results of another, similar, experiment using the same method of fixation. Spectrophotometric assay of the nucleotide in a portion of the muscle gave the value 8·1 m-mole/kg muscle; the radioactivity was 0·29 μc/g muscle per μc/g frog. No control muscle was used, but the figures again indicate that no substantial loss of nucleotide could have taken place.

An autoradiograph of a transverse section of the muscle fixed in lanthanum-free solution shows that the distribution of activity in the individual fibres is uniform over the cross-section (Pl. 2, fig. 1). This is entirely different from the distribution seen (Hill, 1960) after fixation of a muscle in a solution containing lanthanum. The transverse mobility has been lost, as of course was evident also from the fact that the nucleotide did not escape into a non-precipitating fixative.



Text-fig. 2. Distribution of grains in autoradiographs of a sartorius muscle fixed in a lanthanum-free solution. A total of 4367 grains is represented. These are grouped in thirteen blocks between the centre of the I band (arrow at left) and the centre of A (arrow at right). The arrow at B is placed 0·75 μ from A and represents the position of the A-I boundary. The variation expected from random fluctuations in the radioactive emission is indicated by the uppermost and the lowest horizontal lines at the top of each block; these are displaced by 1 S.D. above and below the recorded value. Sarcomere length, 2·71 μ. Muscle section 0·3 μ thick; exposure 8 days.

Part of an autoradiograph of a longitudinal section is shown in Pl. 2, fig. 2. (Attention is drawn to the explanation of this plate where there is a comment on the size of the silver grains.) Analysis of the distribution (Text-fig. 2) shows that a high proportion of the radioactive nucleotide is located at or about the A lines. The analysis was extended to include a large enough number of grains (4367) to confer reasonable significance upon a peak which appeared also at the position of the Z line. If, as before, it is assumed that a disk source produces a 'tail' equal in height to 10% of the peak, at 0·6 μ from the peak, it can be calculated that 78% of the total nucleotide in the muscle is aggregated just inside the A band in the position of the A lines. In addition there is a further deposit at the Z line;

this could not be more than about 10% of the whole, for the remaining 10% has had to be allowed as 'background' in the A band.

The distribution is in contrast with that (Hill, 1960) seen in a muscle fixed in a solution containing lanthanum, where the peak, associated with what was estimated to be 70% of the total nucleotide, was found to be in the position of the N bands.

The identity of the A and I regions of the sarcomere was established by using polarized light, in order to be quite sure that the various features in the stain pattern were correctly referred to the A and I bands.

DISCUSSION

It is undesirable to give too much weight to a particular interpretation of the type of distribution shown in Text-figs. 1, 2. The one which is proposed does not necessarily provide a unique solution because it depends too critically upon the data used here, and previously (Hill, 1959, 1960), for calculating the expected distribution of autoradiographic grains on either side of a 'disk' source running transversely across the muscle. These data have not been tested experimentally. It is not impossible (to take an extreme case) that the resolution is so much greater than has been supposed that the distribution observed can practically be taken to represent the actual distribution of the tritium in the sarcomere. In this event the results would suggest that there is a broad band of nucleotide in the muscle, rather than a highly concentrated aggregate. The change which is observed, as between muscles fixed in media with lanthanum either present or absent, would then have to be interpreted in a less dramatic way. It is implied, on the present basis, that 70 or 80% of the nucleotide in a muscle may undergo a movement from one sharply defined site to another. An alternative view could be that the results show the presence of a broader band of nucleotide in the general region of the A-I boundary, the centre of which undergoes a small displacement in some circumstances.

When the results described in this paper are taken in conjunction with the earlier ones (Hill, 1960) one feels disinclined to speculate very much about the meaning, in physiological terms, of the disparity in the results obtained by the different methods. The N bands and the A lines are sites which are preferred by the adenine nucleotide of muscle under the various conditions of fixation actually employed, and if they may similarly be preferred in certain circumstances in living muscle the problem remains to discover what these circumstances are. What physiological factors are involved in causing the nucleotide molecules to assume one rather than the other locality in living muscle? When lanthanum is present in the fixative a non-uniform distribution of radioactivity is seen in transverse sections

of the muscle, and it has been argued (Hill, 1960) that the nucleotide must be capable, under these conditions, of diffusing in a transverse direction across the muscle fibre. When the muscle is acted upon by fixative not containing lanthanum the mobility is lost; this is proved first by the failure of the nucleotide to escape into the solution, and secondly it is confirmed by the display of an entirely uniform distribution in autoradiographs of transverse sections of the muscle. If lanthanum is absent mobility of the nucleotide is lost; and it is lost irreversibly, for even the subsequent introduction of lanthanum (which was present in the solution used for washing out the fixative) does not restore it. In view of this irreversibility it is perhaps justifiable to argue that what happens when lanthanum is absent is a degradative process. On the whole one inclines to the view that the earlier results, using lanthanum in the fixative, are the more likely to indicate the state in the normal resting muscle. The term 'degradative process' is not meant to imply that the concept of an A-line siting of nucleotide is not of physiological relevance. It is not impossible that the changes which occur in lanthanum-free fixative are analogous with those ensuing from activity of the normal muscle, and that physiological recovery is able to reverse the situation and restore the nucleotide to the N bands. Reactions which are irreversible in the fixative are not necessarily so *in vivo*.

What, then, is implied by the results of the experiment with the m. ext. long. dig. IV? Lanthanum was present, and since degradative processes might be supposed to be slowed or halted by the low temperature at which fixation is assumed to have been effected, it might be hoped that results produced by this method would more nearly represent normality than would any of the others. The crux of the matter is in the role of the lanthanum in the freeze-substitution medium. Can it act as a precipitant by forming an insoluble nucleotide salt when water is absent? If not, the method is no better than one in which a lanthanum-free solution is used for fixing an unfrozen muscle. (There would not appear to be any simple way of testing the reaction of adenine nucleotide with lanthanum in a non-aqueous medium.) The 'degradative process', leading from the N-band to the A-line distribution, though no doubt greatly retarded by the low temperature, might, nevertheless, take place in the time given (6 days) and the end-results by the two methods would be similar. In fact, however, they are comparable only in so far as both experiments show distributions of nucleotide predominantly centred on the A lines. The stain patterns are not identical, for basophilic material is present in the N-band region of the freeze-substituted muscle, but this is not so in the one fixed in the lanthanum-free solution. Is the contrast perhaps connected with the different degrees of aggregation at the A lines in the two cases? Where the N bands

are still present (freeze-substituted muscle) only about 50% of the nucleotide is aggregated at the A lines, compared with about 80% in the other case. The transformation from the N-band to the A-line condition may therefore be incomplete in the former, and this may account for some retention of the N-band pattern. The grain distribution (Text-fig. 1) could not, owing to insufficient resolution, give a definite indication of a subsidiary peak at the N-band region because it would be too close to the main one at the A lines.

The state of phosphorylation of the nucleotide may be the main factor determining the preference of its siting. A new approach to the problem would be by establishing whether a preference of the nucleotide molecules for a particular site was associated with a definite state of phosphorylation. It may be taken for granted that nucleotide precipitated by lanthanum is immune from enzymic dephosphorylation. When lanthanum is present in the fixative does it penetrate into the muscle with sufficient rapidity to precipitate the nucleotide before there has been time for dephosphorylation to occur? Does dephosphorylation occur in lanthanum-free fixative? If questions such as these could be answered it might be possible to establish a correlation between siting and the state of phosphorylation. (Any investigation on these lines might encounter serious trouble when it came to determining the actual state of phosphorylation of the nucleotide, because of the difficulty of extracting the nucleotide from the fixed muscle without altering the degree of phosphorylation.)

Adenine nucleotide is present not only in the acid-soluble fraction, but also in the nucleic acid of muscle. It has been shown (Hill, 1959, 1960) that about 90% of the radioactivity is due to the acid-soluble nucleotide, and some 10% to the nucleic acid. The results obtained so far must therefore show primarily how the acid-soluble nucleotide is sited, and the siting of the nucleic acid must be entirely masked. The basophilic character of some component present at the N bands and A lines suggests these as regions where nucleic acid is located. Further experiments are necessary, using labelled purine or pyrimidine bases represented only in the different forms of nucleic acid, for the purpose of locating their sites in muscle. This will be the subject of later work.

The secondary bands and lines seen in striated muscle must surely be sites where chemical changes occur during the contraction or recovery of muscle. The Z line is thought to be concerned with the inward transmission of the excitatory influence of membrane depolarization (Huxley & Taylor, 1958), but very little, or nothing, is known about the M and N bands or about the A lines. In this connexion it is worth mentioning a promising method, recently described, of investigating the distribution of well-defined protein fractions in sections of muscle. When such a fraction

has specific antigenic properties the corresponding antibody may be prepared, and if this is rendered fluorescent and then allowed to react with the antigen in a muscle section, the microscopical location of the fluorescence indicates the site of the protein fraction in question. Marshall, Holtzer, Finck & Pepe (1959) have discussed the possibilities and limitations of this method in connexion with the location of actin, tropomyosin and of myosin fragments. The myosin fragments are of special interest here. Myosin may be split into two main fractions, the H and L fractions. The antigen of the H fraction is situated largely at or near the M line. The L fraction appears to be sited at the lateral parts of the A-band region. When the L fraction is further broken down a fraction known as L-1 is produced. This is of particular interest because of its unusual properties; it appears, from optical rotation studies (Cohen & Szent-Györgyi, 1957), to be largely α-helical in structure. It has the virtue of consisting almost entirely of a single antigenic species and is therefore well suited to location by the antibody method. Marshall *et al.* (1959) have shown that L-1 is rather sharply confined to the extreme lateral edges of the A band. This is precisely the location of the A lines where, according to the present results adenine nucleotide can, in specified circumstances, become aggregated. It is interesting to note that the ATP-ase activity of myosin is confined to the H fraction and that the L and L-1 fractions have no enzymic properties.

SUMMARY

1. The work described is an extension of an earlier study of the location, by autoradiography, of tritium-labelled adenine nucleotide in sections of resting frog's muscle.

2. The method of freeze-substitution was used for fixing a m. ext. long. dig. IV. Both osmium tetroxide and lanthanum nitrate were dissolved in the ethanol used for substitution.

3. A sartorius muscle was fixed with osmium tetroxide in a solution not containing lanthanum. It was found, unexpectedly, that adenine nucleotide was retained in the muscle despite the absence of any precipitant in the fixative.

4. The autoradiographically determined distribution of nucleotide in the sarcomere showed in both cases that there was aggregation of a high proportion of the nucleotide at the 'A lines' (in the A band, near the boundary with the I band). In the m. ext. long. dig. IV, fixed by freeze-substitution, about 50% of the total activity was concentrated in this region; in the sartorius, fixed in lanthanum-free solution, the proportion was nearly 80%, and in addition there was a significant peak, representing perhaps 10% of the whole, centred on the Z line.

5. The emphasis is primarily on the demonstration of the existence of

preferred sites. The physiological conditions under which the sites become active as centres for nucleotide metabolism are not known.

REFERENCES

COHEN, C. & SZENT-GYÖRGYI, A. G. (1957). Optical rotation and helical polypeptide chain configuration in α-proteins. *J. Amer. chem. Soc.* **79**, 248.

FEDER, N. & SIDMAN, R. L. (1958). Methods and principles of fixation by freeze-substitution. *J. biophys. biochem. Cytol.* **4**, 593–602.

HILL, D. K. (1959). Autoradiographic localization of adenine nucleotide in frog's striated muscle. *J. Physiol.* **145**, 132–174.

HILL, D. K. (1960). The location of adenine nucleotide in frog's striated muscle. *J. Physiol.* **150**, 347–373.

HUXLEY, A. F. & TAYLOR, R. E. (1958). Local activation of striated muscle fibres. *J. Physiol.* **144**, 426–441.

MARSHALL, J. M., Jr., HOLTZER, H., FINCK, H. & PEPE, F. (1959). The distribution of protein antigens in striated myofibrils. *Exp. Cell Res.* Suppl. 7, 219–233.

EXPLANATION OF PLATES

PLATE 1

Fig. 1. A 0·3 μ section of a muscle (m. ext. long. dig. IV) fixed by freeze-substitution. The section was heat-sealed at 180° C for 6 min, and stained for 28 min at 30° C with crystal violet buffered at pH 4. The pattern consists of close-spaced pairs of N bands. Sarcomere length, 2·9 μ.

Fig. 2. A composite photograph of the Z- and A-line pattern at two sarcomere lengths. The sections, 0·5 μ thick, were cut from a sartorius muscle which had been fixed in a lanthanum-free solution. The sections were not heat-sealed. They were stained for 12 min at 20° C with crystal violet buffered at pH 4. The pattern, typical of the greater part of the section, consists of Z and A lines; these are identified by observing that each Z line is flanked by two A lines, making a distinct triplet. Sarcomere lengths, 2·70 and 2·25 μ. The photographs have been set alongside one another to illustrate the point that the distance between pairs of A lines as measured across the A band is the same at the different sarcomere lengths.

Fig. 3. Another part of the section seen in Fig. 2. Only a few fibres in the section stain in this way; the Z and A lines are seen, but in addition the A band, between the A lines, has taken up some stain. Sarcomere length, 2·73 μ.

PLATE 2

Fig. 1. Autoradiograph of a transverse section of a sartorius muscle fixed in a lanthanum-free solution. Section thickness, 0·2 μ; exposure 49 days. Section not stained. The degree of blackening, indicating the amount of radioactivity, is uniform in the individual fibres, showing that the adenine nucleotide is evenly distributed in the cross-section.

Fig. 2. Autoradiograph of a longitudinal section of a sartorius muscle fixed in a lanthanum-free solution. Section thickness, 0·3 μ; exposure 8 days. The section was not heat-sealed; it was stained for 24 min at 20° C with crystal violet at pH 4. Sarcomere length, 2·71 μ. The stain pattern is not the simple one consisting of Z and A lines which is characteristic of this preparation when bare sections are stained; for some reason the presence of the autoradiographic film causes the A band, between the A lines, to stain deeply, and the A lines are masked. The Z lines remain prominent. It should be noted that the images of the autoradiographic grains are, for various reasons, considerably larger than the original silver bromide grains; the latter have a diameter of about 0·2 μ, and the effective 'target' may be even smaller (Hill, 1960). The centres of the grain images may be pin-pointed with accuracy, and it is justifiable to assume that the centre of the original 'target' is thereby located.

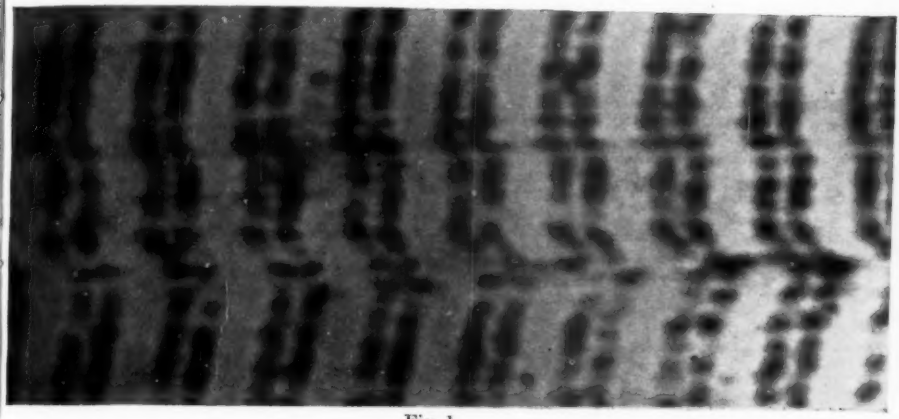


Fig. 1

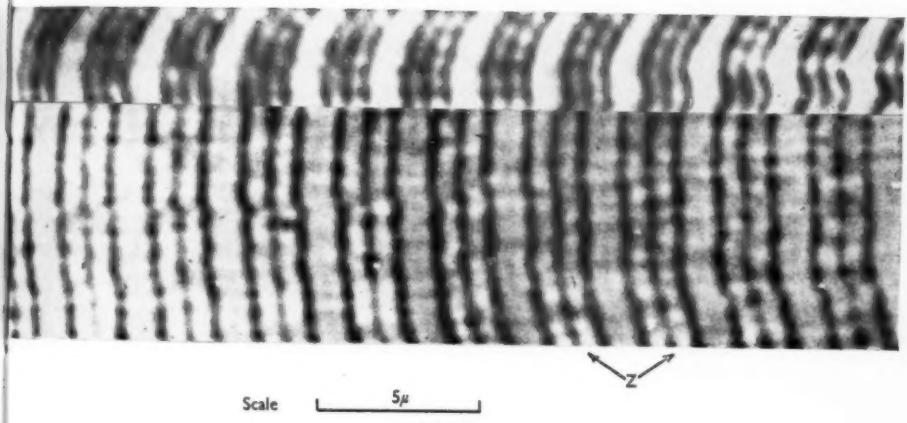


Fig. 2

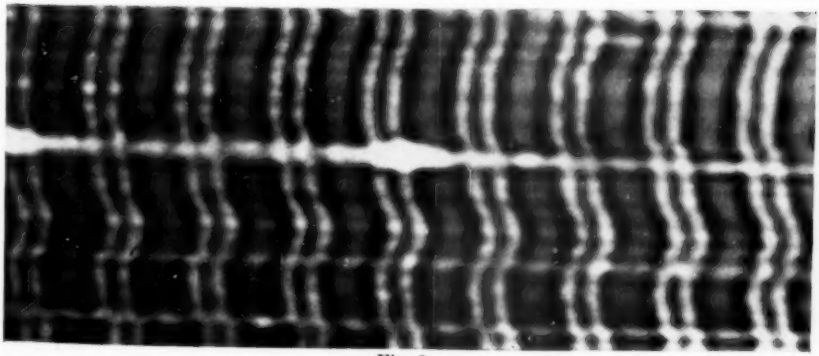
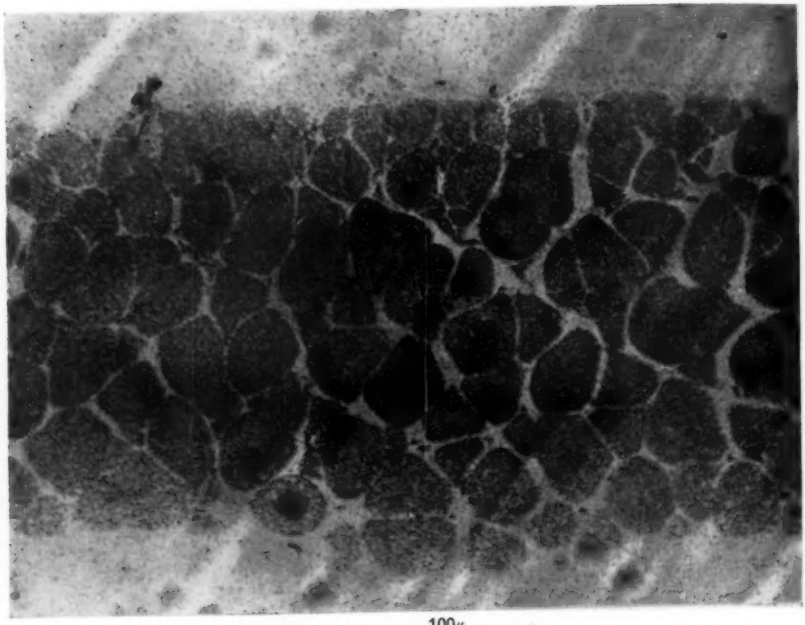


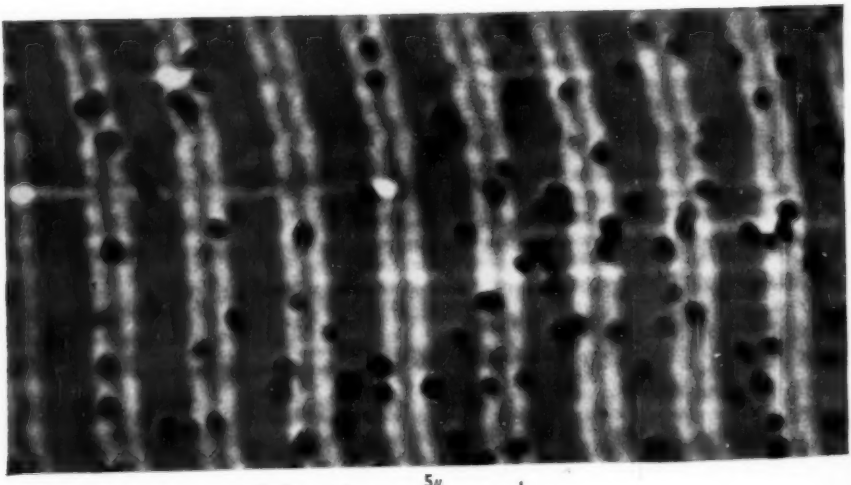
Fig. 3

(*Facing p.* 446)



Scale 100μ

Fig. 1



Scale 5μ

Fig. 2

J. Physiol. (1960), **153**, *pp.* 447–456
With 1 plate and 1 text-figure
Printed in Great Britain

RESPONSES OF FOETAL AND NEW-BORN MONKEYS TO ASPHYXIA

BY H. N. JACOBSON AND W. F. WINDLE

From the Laboratory of Neuroanatomical Sciences, Field Station of Perinatal Physiology, National Institute of Neurological Diseases and Blindness, National Institutes of Health, Public Health Service, U.S. Department of Health, Education and Welfare, San Juan, Puerto Rico, U.S.A.

(*Received* 21 *April* 1960)

Many clinical studies have implicated anoxia before or during birth as a cause of later neurological defects. This is thought to be one of the principal causes of human cerebral palsy and mental retardation, but few efforts have been made to apply laboratory techniques to a solution of the problem (Bailey, 1958). Studies of asphyxia neonatorum in foetal guinea-pigs have been correlated with histological changes in the nervous system (Windle & Becker, 1942, 1943; Windle, Becker & Weil, 1944; Windle, 1958; Bailey & Windle, 1959). Other laboratory species have been used to investigate effects of oxygen deprivation after birth (Ford, 1928; Fazekas, Alexander & Himwich, 1941; Meier & Bunch, 1950; Meier & Menzel, 1955) but without adequate anatomical controls. Yet the differences between human foetuses or new-born infants and these laboratory animals have been considered too great to permit close comparisons to be drawn. The primate should be a more favourable subject, and we have employed the rhesus monkey, *Macaca mulatta*, which has much in common with man in respect to the physiology of reproduction, although its gestation period is shorter (168 days, on the average; van Wagenen, 1958) and the offspring is more advanced in development at birth.

METHODS

Rhesus monkeys were provided from caged colonies. They were mated during one 24-hr period of the menstrual cycle. The maintenance of caged breeding colonies, the practice employed to obtain timed matings, and the procedures used in rearing asphyxiated and normal infants in a nursery have already been described (Jacobson & Windle, 1960).

Foetuses were delivered, without rupturing the foetal membranes, by Caesarean section under local anaesthesia at 159 ± 2 days of gestation. The foetus was asphyxiated by leaving it within the membranes for a variable interval of time after the last gasp. After it had been removed from the membranes, an airway was established by tracheal intubation through a neck incision in the first four experiments, but this was discontinued in favour of endo-

tracheal catheterization in the last ten. A polyethylene catheter with an external diameter of 0·11 in. (2·8 mm) was inserted through the larynx into the trachea under direct vision; it was anchored by a thread to the lower lip. A rubber bag containing oxygen was attached to the catheter and the foetus was resuscitated by intermittently inflating the lungs at a rate of approximately 50/min, and at pressures of 30–42 mm Hg. Ventilation was controlled manually so that administration of oxygen could be adjusted to correspond to the new-born infant's own pattern of respiration, once this reappeared. Resuscitation was discontinued when the new-born infant was able to breathe by itself. Twelve foetuses were successfully resuscitated and survived for at least 24 hr.

Seven new-born monkeys 1–2 days old, and one of 158 days' conceptual age, were asphyxiated by placing them in a glass jar through which nitrogen was flowing. Of these eight monkeys, two were delivered by Caesarean section at 157 and 158 days, five were born vaginally at term, and one was born vaginally at 148 days, but was reared to a conceptual age of 158 days to correspond with the other new-born monkeys. The time to last gasp was measured. In four experiments the period of asphyxia was continued beyond the last gasp. In four other experiments resuscitation was begun as soon as possible after the last gasp.

The duration of asphyxia was measured from the time at which the placenta was separated from the endometrium, or at which an infant monkey was placed in nitrogen, until the moment when oxygen was passed into the lungs. The foetal heart rate was counted either from pulsating vessels, where visible, or from an electrocardiogram. Unasphyxiated monkeys of similar ages were also used for studies of behaviour and for histological examination of the central nervous system. Asphyxiated surviving monkeys and controls were killed by perfusion-fixation through the ascending aorta, with drainage from the right atrium and inferior vena cava (Kcenig, Groat & Windle, 1945; Cammermeyer, 1960).

RESULTS

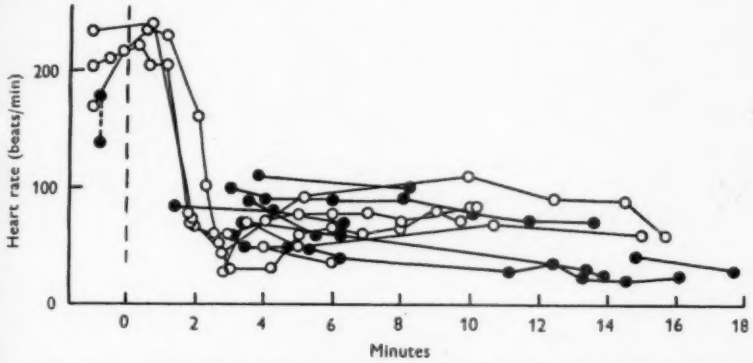
Activity during asphyxia. Eight of the twelve foetuses delivered within the membranes (and thus asphyxiated) responded briefly to the mechanical stimuli of delivery by moving their limbs and trunks; on one occasion sucking was seen. As soon as handling stopped, the foetus in its membranes became quiescent. Later strong rhythmical mass movements occurred in some foetuses for a few seconds only. No meconium was passed. Respiratory efforts began 153 ± 38 (S.D.) sec after placental separation. They usually occurred at a frequency of 5–6/min, but frequencies up to 20/min were encountered twice. The respiratory movements first increased in amplitude, then gradually became weaker, and after lasting for an average of 6 min 50 sec finally ceased.

New-born monkeys placed in nitrogen struggled for air, defaecated, salivated, and vocalized for the first 3–4 min, after which they became comatose and apnoeic. After a little while gasping began and continued, on the average, for a further 5 min 42 sec.

Heart rate. The heart rate of the anaesthetized unasphyxiated monkey foetus at Caesarean section is 140–170 beats/min (Dawes, Jacobson, Mott & Shelley, 1960). Rates of 100–180 beats/min have been recorded through the intact abdomen during the last third of gestation (Hartman, Squier & Tinklepaugh, 1930). That of the unanaesthetized new-born

monkey is 205 ± 20 (S.D.) beats/min. The heart rate of seven foetuses in the present experiments declined slowly and progressively during asphyxia (Text-fig. 1). The average heart rate after 3–4 min was 86 beats/min (range 60–108): after 6–7 min it had fallen to 67 beats/min (range 42–92) and continued to decline through the remainder of the asphyxiation.

Another foetus was atypical. Electrodes were applied to the foetal membranes 4 min after delivery, when the heart rate was only 36/min. This was a rate ordinarily encountered only after 7–8 min asphyxia (Text-fig. 1). Furthermore, the foetus could not be aroused and made no respiratory efforts after delivery from the uterus. This could be explained by a period of hypoxia before the experiments, though there was no direct evidence of this.



Text-fig. 1. The course of heart rate changes during asphyxia in foetal (●, 7) and new-born (○, 5) monkeys; the number of animals in each group is shown in brackets. The vertical broken line indicates time of onset of asphyxia. Range of heart rates in the foetus before asphyxiation is from Hartman *et al.* (1930).

The effect on heart rate was different in new-born monkeys placed in nitrogen. At first there was a slight increase in the average heart rate, from 202 to 226 beats/min, followed by a sharp fall to 52 beats/min (range 30–72). This occurred between 1 and 3 min after the start of nitrogen breathing (Text-fig. 1) and coincided with salivation, defaecation, and struggling. After the initial fall the heart rate gradually increased to 88 beats/min (range 78–108) between 9 and 10 min after the start of anoxia. Thereafter the rate declined.

Resistance to O_2 lack. The resistance to anoxia may be measured by the longest period of oxygen lack to which a foetus or new-born infant can be subjected and, when resuscitated, survive for at least 24 hr. At first, the time at which resuscitation was begun was based on the supposition that the last gasp closely anticipated the limit of tolerance of the foetus. There-

fore, in ten experiments, the foetus was removed from the membranes when its respiratory efforts had ceased, and was resuscitated as quickly as possible. The time from the last gasp to the onset of resuscitation varied from 2 min 52 sec to 6 min 54 sec (Table 1), and it was clear that the time of the last intra-amniotic gasp did not signify that the end of tolerance was imminent, since all of these monkeys were resuscitated and survived at least 24 hr. In the next two experiments the foetus was removed from the membranes as usual and prepared for resuscitation, but asphyxia was

TABLE 1. Resistance of foetal monkeys to asphyxia

Monkey no.	Duration of asphyxia	Time to last gasp	Time, last gasp to resuscitation with O_2	Time, resuscitation to spontaneous breathing
	min sec	min sec	min sec	min sec
	A Survived for 24 hr			
6	11 11	8 19	2 52	6 19
2W	13 30	10 16	3 14	9 10
65	11 33	8 13	3 20	8 27
60	14 05	10 44	3 21	10 55
66	13 15	8 54	4 21	8 45
31	17 00	12 20	4 27	13 00
57	11 58	7 03	4 55	12 02
26	15 50	10 35	5 15	10 00
69	16 00	10 39	5 21	14 10
33	16 20	10 52	5 28	17 00
76	14 15	8 47	5 28	14 25
63	13 25	6 31	6 54	13 05
Average	14 02	9 26	4 37	11 26
	B Did not survive 24 hr			
80	16 33	6 14	10 19	120 (approx.)
71	21 30	8 29	13 01	No gasps

continued until the heart rate had diminished to 30 beats/min. The times from the last gasp to resuscitation were 5 min 21 sec and 5 min 28 sec, and both survived. Resuscitation was delayed further in two more experiments. The asphyxia was continued for 4 min after the heart rate had been reduced to 30 beats/min; the intervals between the last gasp and resuscitation were 10 min 19 sec and 13 min 1 sec (Table 1*B*): these two monkeys did not recover.

The experiments on new-born infants are illustrated in Table 2. In four monkeys (Table 2*B*), the intervals between the last gasp and resuscitation (2 min 30 sec—6 min 24 sec) were comparable with those of surviving foetuses (Table 1), but the new-born monkeys did not recover. Four other monkeys (Table 2*A*) were removed from the nitrogen after a gasp that was considered to be the last. Three of these made feeble inspirations after removal from the nitrogen and before oxygen was administered. The other monkey was resuscitated 1 min 29 sec after its last gasp. All four survived.

Thus all the foetal monkeys asphyxiated in their membranes survived anoxia for longer periods of time than the new-born monkeys placed in nitrogen.

Recovery from asphyxia. Asphyxiated foetuses started breathing after intervals varying from 6 min 19 sec to 17 min from the onset of resuscitation (Table 1). This period showed a direct correlation with the interval between the last gasp and the initiation of resuscitation ($r = 0{\cdot}83$, $P < 0{\cdot}01$). By contrast, in the four surviving new-born monkeys spontaneous respiratory movements occurred within 1 min after oxygen entered the lungs (Table 2). In those foetal and new-born monkeys which were successfully resuscitated the heart rate began to recover almost immediately after oxygen administration started.

TABLE 2. Resistance of new-born monkeys to asphyxia

Monkey no.	Duration of asphyxia	Time to last gasp	Time, last gasp to resuscitation with O_2	Time, resuscitation to spontaneous breathing
	min sec	min sec	min sec	min sec
	A Survived for 24 hr			
79	8 00	8 00	0 00	0 00
93	10 15	10 15	0 00	0 00
92	10 50	9 36	1 14	1 00
91	10 39	9 10	1 29	0 46
	B Did not survive 24 hr			
1W	13 35	11 05	2 30	No gasps
77	13 02	10 02	3 00	4 58
90	16 05	10 20	5 45	No gasps
87	14 22	7 58	6 24	No gasps

Later effects of anoxia. There were many differences between the behaviour of all the monkeys asphyxiated *in amnio* and that of normal animals delivered by Caesarean section, as observed during the weeks or months after birth. These suggested that the asphyxiated foetuses had suffered cerebral damage, and detailed descriptions of the behavioural changes are being published elsewhere (Ranck & Windle, 1959; Robert de Ramirez de Arellano, McCroskey, Dennery & Windle, 1959; Saxon, 1960*a*, *b*; Windle, Jacobson, Robert de Ramirez de Arellano & Combs, 1960). New-born monkeys resuscitated after breathing nitrogen recovered rapidly. There were no apparent differences between them and normal infants of comparable ages.

The successfully resuscitated infants (which had been asphyxiated as foetuses) and controls were usually killed for histological studies 3–14 days after asphyxia (one was killed 10 months later). Structural changes in the central nervous system were encountered in all the asphyxiated monkeys

examined histologically. The lesions of some of these have been described elsewhere (Ranck & Windle, 1959). In five serially sectioned specimens a bilaterally symmetrical pattern of neuronal loss and neurological reaction, conforming to nuclear boundaries, was observed. The nuclei of auditory, vestibular, and somatic afferent pathways, including the thalamus, were usually severely injured, with maximal effects appearing in the inferior colliculi (Pl. 1*A*). In some the cerebral cortex, hippocampus, cerebellum, and spinal cord were also involved. Haemorrhages related to asphyxia were not found.

The monkeys which survived exposure to nitrogen after birth were killed 10–13 days later. The structure of the central nervous system of these animals was strikingly different from that of monkeys asphyxiated *in amnio*. No focal lesions were encountered in the brain stem, although the possibility of some diffuse neuronal loss could not be ruled out with the methods used (Pl. 1*C*, *D*). The cerebral cortex, hippocampus, cerebellum, and spinal cord appeared to be free from defects, resembling the control specimens in all respects.

DISCUSSION

The length of the interval of time during which a monkey foetus, undergoing asphyxiation within its membranes, could be successfully resuscitated was variable. Intra-amniotic respiratory movements started at different times after placental separation; indeed, they did not appear in one foetus that was considered to be moribund *in utero*. Probably their occurrence is related to declining oxygen saturation of the foetal blood in the monkey, as it is in other species of animals (Windle, 1940). Lacking any estimation of the foetal oxygen reserve at the moment of interruption of the placental exchange, we could not predict the length of time the foetus would survive before resuscitation became imperative.

When asphyxiation was terminated at or just before the time of the last gasp within the foetal membranes, resuscitation was not required. However, when resuscitation was delayed further, until the foetal pulse had slowed to 30 beats/min or less for a period of 4 min, the monkey foetuses failed to survive. The last time, compatible with life, at which resuscitation must be started is between 7 and 10 min after the last gasp within the membranes. These observations enable one to make a closer estimation of the limit of tolerance of foetal monkeys to asphyxiation than could have been obtained by arbitrarily chosen time limits. Had 10 min been selected, for example, as the terminal limit of asphyxiation within the foetal membranes, monkeys 2W, 60, 31, 26, 69, and 33 would have gasped air spontaneously upon breaking the amnion.

How long a period of asphyxia a foetal monkey might tolerate without

cerebral defects being produced has not been determined. The average interval of time after the last gasp of surviving monkeys was 4 min 37 sec; all of them were damaged, several of them severely, and histological studies revealed brain lesions. It is hardly to be believed that monkey 57 (Windle *et al.* 1960) could have survived more extensive neural tissue damage than it received, even though resuscitation was started after the average lapse of time. On the other hand, in some of the experiments in which greater than average lapses of time occurred the monkeys appeared to have been less severely affected neurologically.

In the new-born monkeys which were given nitrogen, resuscitation was ineffectual when begun more than 2·5 min after the last gasp. The decline in heart rate was more precipitous than that encountered in the foetus. Does the heart of the new-born and young monkey require more oxygen or has it fewer metabolic reserves to call upon than that of the foetus? Shelley (1960) and Dawes *et al.* (1960) observed that the glycogen content of the foetal heart muscle was higher initially and was still measurable 2 min after the last gasp, whereas that of the post-natal monkey breathing nitrogen was almost wholly depleted at a corresponding time. The foetus consumes oxygen at a slower rate than the new-born animal. Furthermore, blood pressure and blood glucose respond differently in asphyxiated foetal and new-born monkeys (Dawes *et al.* 1960). These differences reflect fundamental changes taking place in early post-natal life, about which we have little knowledge at present. The initial higher glycogen reserve may help explain the greater resistance of foetal monkeys to asphyxiation.

From the present experiments it is clear that the critical point for resuscitation of the new-born monkey after breathing nitrogen was considerably closer to the time of the last gasp—between 1 and 3 min—than in the foetus. No striking neurological nor histological defects resulted in the new-born animal from the procedure. This is another indication of difference between the two. Perhaps sensitivity of the heart to anoxia in the new-born monkey precluded the infant's survival for a period of time adequate to produce neural damage. In other words, it might be that the heart of the new-born monkey is less resistant than the brain, but that the reverse is the case in the monkey foetus.

It was unnecessary to prolong asphyxiation of the foetus beyond the end of intra-amniotic respiratory movements to induce neurological and histological defects. Changes have been observed in one that had been asphyxiated for 11 min but required no resuscitation (Windle, unpublished). In contrast, the new-born monkeys asphyxiated for 1 min or more after the last gasp in nitrogen showed no clear defects. It would seem that 10–11 min may be the critical length of time for which the monkey's

central nervous system can withstand oxygen deprivation at the end of gestation without apparent neural tissue damage.

How this compares with conditions in human foetuses and infants is unknown. There are marked species differences. New-born rats placed in nitrogen continue to execute respiratory movements for 50 min; kittens and puppies, 25–28 min; new-born rabbits, 17 min; and guinea-pigs only about 7 min (Fazekas *et al.* 1941). Nothing is known about the effects of such procedures on the central nervous system of these animals.

One cannot determine the degree of asphyxiation of a foetus at birth simply by inspection. Apnoea is not a reliable indication of severe oxygen want. Even in the absence of narcosis, the foetus is normally apnoeic before and during the early stages of a declining oxygen supply. The phenomenon of arousal is observed occasionally during mechanical stimulation, but the individual resumes quiescence when stimulation ceases (Windle, 1940). Oxygen want also induces arousal; moreover, it brings on rhythmical mass movements, including respirations in the form of gasps. The 'apnoea' that ensues when gasping ceases is asphyxial; but it may not be easy to distinguish it from physiological apnoea. Clinical studies in which attempts are made to employ the interval of time between birth and onset of spontaneous respiration as an index of anoxia ignore these facts. The physiologically apnoeic (often narcotized) human foetus, capable of initiating respiratory activity without assistance by the resuscitator, is not an asphyxiated foetus. It should not be expected to exhibit neurological and psychological defects because it was found to be apnoeic. Arousal requires normally functional nervous centres, the thresholds of which may be affected by drugs, which can be avoided in experiments on monkeys by performing operations under local anaesthesia.

SUMMARY

1. The responses of foetal monkeys asphyxiated in their intact membranes and of new-born monkeys asphyxiated in nitrogen are presented, together with estimates of their resistances to anoxia.

2. Foetal monkeys within intact membranes were active for a short time after being delivered; thereafter they were quiescent except for gasping respirations during asphyxia. The foetal heart rate declined slowly during anoxia. They were successfully resuscitated as long as 6 min 54 sec after the last intra-amniotic gasp.

3. New-born monkeys in an atmosphere of nitrogen struggled violently, defaecated and salivated. The heart rate fell precipitously, then rose gradually before ultimately declining. Resuscitation begun 2·5 min or more after the last gasp was not successful.

4. Brains of monkeys asphyxiated either within their membranes or in nitrogen after birth were examined histologically at various times after asphyxia. Severe, symmetrical, focal destruction was seen in brains of monkeys made anoxic as foetuses. Brains of new-born monkeys asphyxiated in nitrogen were not similarly affected.

Many people participated in the experiments from which data reported in this communication were drawn. Surgical and physiological experiments were carried out with Drs C. J. Bailey, C. M. Combs, J. G. Frontera, L. Guth, D. L. McCroskey, C. A. Pfeiffer, Marisa I. Robert de Ramirez de Arellano, J. B. Ranck, Jr., Miss Sue V. Saxon, and Dr W. Stiehl assisted by Mrs Elizabeth Dorrill, Mr O. Nieves, and Mr J. O. Smart.

REFERENCES

BAILEY, C. J. (1958). Interrelationship of asphyxia neonatorum, cerebral palsy and mental retardation: present status of the problem. In *Neurological and Psychological Deficits of Asphyxia Neonatorum*, ed. WINDLE, W. F., pp. 5–30. Springfield, Illinois: Thomas.

BAILEY, C. J. & WINDLE, W. F. (1959). Neurological, psychological, and neurohistological defects following asphyxia neonatorum in the guinea pig. *Exp. Neurol.* **1**, 467–482.

CAMMERMEYER, J. (1960). The post-mortem origin and mechanism of neuronal hyperchromatosis and nuclear pyknosis. *Exp. Neurol.* **2** (in the Press).

DAWES, G. S., JACOBSON, H. N., MOTT, J. C. & SHELLEY, H. J. (1960). Some observations on foetal and new-born rhesus monkeys. *J. Physiol.* **152**, 271–298.

FAZEKAS, J. F., ALEXANDER, F. A. D. & HIMWICH, H. E. (1941). Tolerance of the newborn to anoxia. *Amer. J. Physiol.* **134**, 281–287.

FORD, F. R. (1928). An experimental investigation into the effects of asphyxia on the brain, with especial reference to asphyxia neonatorum. *Johns Hopk. Hosp. Bull.* **42**, 70–76.

HARTMAN, C. G., SQUIER, R. R. & TINKLEPAUGH, O. L. (1930). The fetal heart rate in the monkey (*Macacus Rhesus*). *Proc. Soc. exp. Biol., N.Y.*, **28**, 285–288.

JACOBSON, H. N. & WINDLE, W. F. (1960). Observations on mating, gestation, birth, and postnatal development of *Macaca malatta*. *Biol. Neonatorum* (in the Press).

KOENIG, H., GROAT, R. A. & WINDLE, W. F. (1945). A physiological approach to perfusion-fixation of tissues with formalin. *Stain Tech.* **20**, 13–22.

MEIER, G. W. & BUNCH, M. E. (1950). The effects of natal anoxia upon learning and memory at maturity. *J. comp. physiol. Psychol.* **43**, 436–441.

MEIER, G. W. & MENZEL, E. W. (1955). Prenatal oxygen deprivation and subsequent specific behavior dysfunctions. *Science*, **122**, 419–420.

RANCK, J. B., JR. & WINDLE, W. F. (1959). Brain damage in the monkey, *Macaca mulatta*, by asphyxia neonatorum. *Exp. Neurol.* **1**, 130–154.

ROBERT DE RAMIREZ DE ARELLANO, M. I., MCCROSKEY, D. L., DENNERY, J. M. & WINDLE, W. F. (1959). Neurological deficits of asphyxia neonatorum in monkeys. *Trans. Amer. neurol. Ass.* **84**, 151–154.

SAXON, S. V. (1960*a*). Effects of asphyxia neonatorum on behavior in the rhesus monkey. *J. genet. Psychol.* (in the Press).

SAXON, S. V. (1960*b*). Differences in reactivity between asphyxial and normal rhesus monkeys. *J. genet. Psychol.* (in the Press).

SHELLEY, H. J. (1960). Tissue carbohydrate reserves in foetal and new-born lambs and rhesus monkeys. *J. Physiol.* **151**, 24–25*P*.

VAN WAGENEN, G. (1958). Breeding and care of monkeys. In *Neurological and Psychological Deficits of Asphyxia Neonatorum*, ed. WINDLE, W. F., pp. 274–284. Springfield, Illinois: Thomas.

WINDLE, W. F. (1940). *The Physiology of the Fetus*. Philadelphia: Saunders.

WINDLE, W. F. (1958). Brain damage in the guinea pig after asphyxia neonatorum. In *Neurological and Psychological Deficits of Asphyxia Neonatorum*, ed. WINDLE, W. F., pp. 31–43. Springfield, Illinois: Thomas.

WINDLE, W. F. & BECKER, R. F. (1942). Effects of anoxia at birth on central nervous system of the guinea pig. *Proc. Soc. exp. Biol., N.Y.*, **51**, 213–215.

WINDLE, W. F. & BECKER, R. F. (1943). Asphyxia neonatorum. An experimental study in the guinea pig. *Amer. J. Obstet. Gynec.* **45**, 183–200.

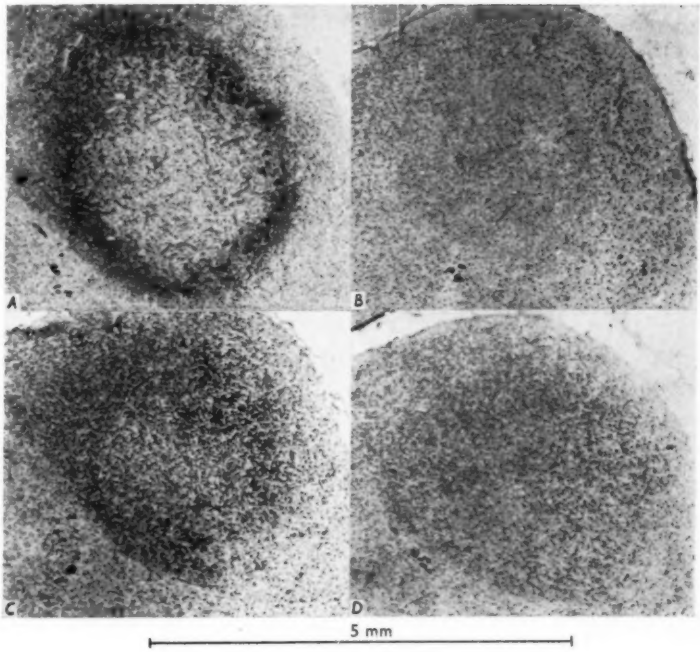
WINDLE, W. F., BECKER, R. F. & WEIL, A. (1944). Alterations in brain structure after asphyxiation at birth. *J. Neuropath.* **3**, 224–238.

WINDLE, W. F., JACOBSON, H. N., ROBERT DE RAMIREZ DE ARELLANO, M. I. & COMBS, C. M. (1960). Structural and functional sequelae of asphyxia neonatorum in monkeys (*Macaca mulatta*). *Res. Publ. Ass. nerv. ment. Dis.* (in the Press).

EXPLANATION OF PLATE

PLATE 1

Nucleus of the inferior colliculus: *A*, *B*, monkeys asphyxiated as foetuses; *C*, *D*, as newborn infants. Global necrosis in *A* is characteristic of inferior collicular lesions of foetuses asphyxiated in their membranes and killed 9 days later. At 10 months, as seen in *B*, gliosis has replaced the necrotic lesion. Many other nuclei were affected by asphyxia. Nitrogen asphyxiation failed to produce these lesions in *C* and *D*. Thionine stain.


A
B
C
D
5 mm

J. Physiol. (1960), **153**, *pp.* 457–472
With 8 text-figures
Printed in Great Britain

THE SURVIVAL OF YOUNG RATS IN NITROGEN

BY ANNE STAFFORD* AND JOSEPHINE A. C. WEATHERALL†
From the Nuffield Institute for Medical Research, University of Oxford

(*Received* 2 *May* 1960)

Young animals that are born blind and relatively immobile can survive for a considerable period in the absence of oxygen. This resistance to anoxia decreases rapidly with increasing age, and reaches adult values within a few days to three weeks after birth. It has been established that glycolysis provides energy for anaerobic survival in young (Himwich, Bernstein, Herrlich, Chesler & Fazekas, 1942; Hicks, 1953) and foetal animals (Dawes, Mott & Shelley, 1959). The investigations of Himwich and his colleagues have been concerned mainly with the resistance of the brains of young animals to oxygen lack (Chesler & Himwich, 1944). More recently, Dawes *et al.* (1959) showed that the survival of foetal lambs after tying the umbilical cord is related to the amount of glycogen stored in the heart, and suggested that one of the limiting factors in anaerobic survival is the ability of the heart to maintain the circulation. There was also a direct correlation between the initial levels of carbohydrate in the hearts of new-born rats, rabbits and guinea-pigs and the times for which gasping movements persisted in nitrogen (Dawes *et al.* 1959). The experiments reported in this paper were designed to investigate the importance of the glycogen in the hearts of new-born rats for their survival in nitrogen, and to attempt to define more clearly the factors determining the anaerobic survival of new-born animals.

METHODS

Animals. Parturition in a rat usually lasts about 2 hr. During this time the first-born rats become cold. After labour is complete, the mother gathers all the rats into a nest and they start to feed. The temperature of the nest is 31–33° C. The number of animals in a litter varies from about eight to fifteen. The weight of a new-born rat is 4–5·5 g; the animals in the larger litters usually weigh less than the animals in the smaller litters. Before experiments the young rats were removed from the nest and were kept in a box maintained at 33° C. Except in fasting experiments, rats were used within 2 hr of removal from the nest Rats of either sex and from 0 to 18 days of age were used.

Exposure to nitrogen. Rats were placed in a double-walled glass tube. Water from a thermostatically controlled bath was pumped between the walls of the tube so that the temperature within the tube was constant to within $\pm 0{\cdot}2°$ C. Air or nitrogen passed through the tube was humidified by being bubbled through water and warmed by being passed through coils in a water-bath. Nitrogen, as supplied commercially, was passed through at about 6 l./min

* Charles Gilbert Heydon Travelling Fellow of the University of Sydney.
† Personal grant from the Sir Halley Stewart Trust.

initially, and at about 2 l./min after 2 min. Samples of nitrogen from the cylinder and of the effluent gas, collected after passing nitrogen through the tube for 2 min, were analysed by Haldane's method, and contained less than 0·1 % oxygen. During exposure to nitrogen the rats were watched through the walls of the tube. The time between starting nitrogen and the last breath was recorded. The heart rate was recorded electrically from two fine wires looped round the thorax and lower abdomen, making contact through electrode jelly. The rat was placed in the tube and the electrode wires were led through the gas outlet to an amplifier which drove a pen writer and a loudspeaker. The heart rate was counted at intervals before and during exposure to nitrogen.

Biochemical estimations. Tissues were extracted in 5 % trichloroacetic acid containing 0·1 % Ag_2SO_4 (Kemp & Kits van Heijningen, 1954) and total carbohydrate in the deproteinized extracts was measured by the method of Mendel, Kemp & Myers (1954). As this method measures glycogen, glucose-1-phosphate, glucose and fructose, losses arising from glycogenolysis during sampling were reduced. Hearts were removed from the rats within 15 sec after decapitation. If other tissues were taken from the same animals, the sampling was completed within 60 sec. For estimation of total lactic acid production rats were decapitated and the head and body homogenized in deproteinizing fluid in a M.S.E. high-speed blender. Lactic acid was measured in the deproteinized extract by the method of Barker & Summerson (1941). Blood was collected on a siliconed watch-glass. The volume was measured in a graduated 0·1 ml. pipette which had been rinsed with heparin-fluoride solution (0·4 % heparin, 8 % sodium fluoride). Volumes between 0·04 and 0·10 ml. could be obtained from new-born rats. Blood was deproteinized with 5 % $ZnSO_4 . 7H_2O$ and 0·3 N-NaOH. Glucose was measured by the glucose oxidase method of Huggett & Nixon (1957) and lactic acid by the method of Barker & Summerson (1941).

The figures quoted for survival times and for biochemical estimations are expressed as mean ± standard error throughout.

RESULTS

The behaviour of new-born rats in nitrogen

Shortly after new-born rats (< 1 day old) were placed in nitrogen their respiration became rapid and shallow. They struggled a little, but did not have convulsions; there was a pronounced fall in heart rate (Fig. 8). From 2 to 20 min after exposure to nitrogen breathing became irregular and sometimes intervals as long as 4 min were seen between gasps. From about 20 min onwards, gasps became more frequent (1–2/min) and reached a maximum rate of about 12/min which persisted for only 30–60 sec. They stopped abruptly. After this there were no further respiratory movements. Rats taken out of nitrogen during this final phase of rapid gasping recovered, apparently completely. Rats left in nitrogen until 15 sec after breathing had stopped did not breathe again. The survival time of a new-born rat in nitrogen was about 28 min at an environmental temperature of 36° C. Electrical activity in the heart was measurable for 5–10 min after the last breath. As the last breath could be timed with reasonable precision, this was used as an end point for survival. At lower environmental temperatures (20° C) the terminal acceleration of breathing was not so clear, but in experiments at 36° C it was seen in all rats.

Rats 16 days old had convulsions from about 15 to 30 sec after exposure to nitrogen. There was then a period of apnoea lasting 30 sec or more, which

was followed by gasps which increased in rate and decreased in depth for the last 15 sec. The mean survival time (to the last gasp) was 2·5 min at 36° C. Rats of 2, 4 and 8 days of age behaved like new-born rats, but the duration of each phase became shorter with increasing age.

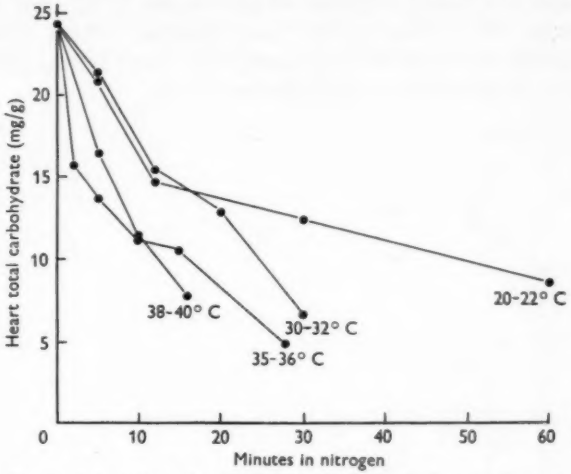


Fig. 1. Rates of depletion of total carbohydrate in the hearts of new-born rats exposed to nitrogen at different environmental temperatures. Each point is the mean of two to four observations. The last point is at the mean time to last breath.

Changes in blood and tissue carbohydrate and lactic acid in new-born rats exposed to nitrogen

Figure 1 shows the decrease of heart carbohydrate in rats less than 24 hr old which were placed in nitrogen at different environmental temperatures. The time to last breath decreased as the temperature of the chamber was increased; heart carbohydrate fell more rapidly at higher temperatures. In all the following experiments in this series rats were exposed to nitrogen at 35–36° C.

Changes in blood glucose and the carbohydrate concentrations in various tissues were measured in rats less than 24 hr old exposed to nitrogen. Preliminary experiments indicated a wide variation in the initial concentration of liver carbohydrate, which ranged from 4 to 100 mg/g in rats less than 24 hr old. On further investigation it became apparent that the concentration of carbohydrate in the liver was related to the time after birth. Figure 2 shows that the concentration of liver carbohydrate in two litters of unasphyxiated rats at birth ranged from 50 to 90 mg/g, but fell rapidly during the first 18 hr of life. This fall occurred although the young rats were suckled, and milk was visible in their stomachs through the

body wall. On exposure to nitrogen a rise in blood glucose was found in rats which had initially a high liver carbohydrate, but only a progressive fall in blood glucose in rats with a low liver carbohydrate. For instance, the mean liver carbohydrate in two litters of rats less than 4 hr old was $58 \pm 7{\cdot}7$ mg/g and during exposure of litter-mates to nitrogen this fell to $36 \pm 3{\cdot}4$ mg/g. The blood glucose concentration, which was initially $73 \pm 8{\cdot}5$ mg/100 ml., rose during anoxia to $116 \pm 7{\cdot}2$ mg/100 ml. and remained at about this level until the last breath.

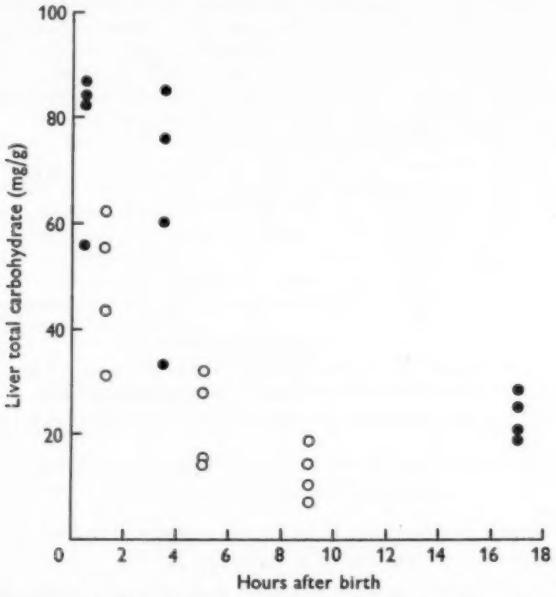


Fig. 2. The concentration of carbohydrate in the livers of new-born rats from two litters (○, litter A; ●, litter B) killed at different times during 18 hr after birth.

In three litters of rats 18–24 hr old the concentration of liver carbohydrate, which was initially much lower than in the younger rats, fell during exposure to nitrogen by only about 4 mg/g (Fig. 3). The blood glucose, initially $63 \pm 6{\cdot}9$ mg/100 ml., fell during exposure to nitrogen to $23 \pm 5{\cdot}2$ mg/100 ml. The total carbohydrate in the heart was almost depleted but in skeletal muscle, lung and skin fell only slightly during anoxia (Fig. 3). As the mean survival time (28·6 min) of litter-mates of ten rats with a low initial liver carbohydrate (< 10 mg/g) was not significantly different ($P > 0{\cdot}9$) from that (28·4 min) of litter-mates of fifteen rats with a high initial liver carbohydrate (mean 51 mg/g), it appears that the ability to mobilize liver glycogen was not of great importance for survival in nitrogen.

The changes in concentrations of lactic acid and of heart and liver carbohydrate in new-born rats before and after exposure to nitrogen are summarized in Table 1. From this it will be seen that during anoxia lactic acid in the bodies of rats less than 12 hr old increased from 0·24 to 1·78 mg/g; this amounted to a change of +7·3 mg in a rat weighing 4·7 g. Concentrations of carbohydrate in the heart and liver fell by about the same amount (21–22 mg/g) but the liver weighed about eight times as

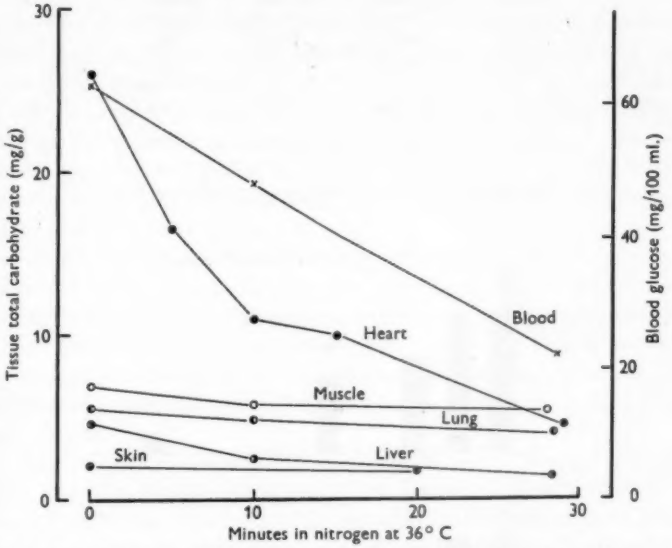


Fig. 3. Concentrations of blood glucose and tissue carbohydrate in new-born rats (18–24 hr old) after exposure to nitrogen for 10 or 20 min or until the last breath. Each point is the mean of four to ten observations (× blood glucose; ● heart; ○ skeletal muscle; ◐ lung; ◑ liver; ⊙ skin).

much as the heart. The loss of carbohydrate from the liver and heart was estimated as 5·2 mg and could account for 72 % of the increase in lactic acid. These results suggested that the carbohydrate mobilized from the livers of young rats during anoxia was being metabolized to lactic acid, but further experiments seemed necessary to determine the relative significance of heart and liver carbohydrate as energy reserves for anoxic survival.

Changes in survival time in nitrogen and in heart and liver carbohydrate with age

The survival times in nitrogen and the initial concentrations of carbohydrate in the hearts and livers of rats from 0 to 16 days of age are shown

in Fig. 4. It will be noticed that the large fall in liver carbohydrate during the first 24 hr after birth was accompanied by only small changes in the concentration of heart carbohydrate and in the survival time. After the

TABLE 1. Mean concentrations of lactic acid in whole rats, and of carbohydrate in hearts and livers of rats less than 12 hr old before anoxia, and of litter-mates exposed to nitrogen until last breath

	Before anoxia (mg/g)	After exposure to nitrogen (mg/g)	Change in concentration (mg/g)	Mean tissue weight (g)	Total change (mg)	
Whole body lactic acid	0·24 (7)	1·78 (9)	+1·56	4·7	+7·3	
Heart carbohydrate	26 (7)	4·7 (13)	−21·3	0·03	−0·64	−5·2
Liver carbohydrate	58 (8)	36 (8)	−22	0·21	−4·6	

The figures in brackets indicate the number of rats.

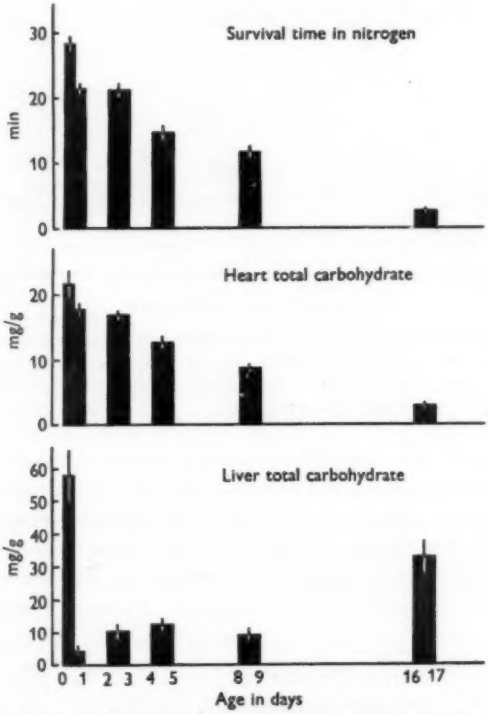


Fig. 4. Concentrations of carbohydrate in the hearts and livers of unasphyxiated new-born rats and the survival times in nitrogen of their litter-mates during 17 days after birth (each block shows the mean and S.E. of ten observations).

first day of life, the concentration of carbohydrate in the liver began to rise, until in 16-day-old rats it had risen to more than half the concentration at birth. At this age the concentration of carbohydrate in the heart and the survival time in nitrogen had fallen to the lowest levels observed in these experiments.

The effect of fasting on heart and liver carbohydrate and on survival time in nitrogen

Rats up to 6 hr old were removed from their nests and kept in a humidified box maintained at 33 ± 1° C. Preliminary experiments showed that control of temperature was essential, as the body temperature (measured with a rectal thermocouple) followed the environmental temperature closely and the carbohydrate in the tissues disappeared more slowly at lower temperatures. The concentrations of carbohydrate in the heart and liver and the survival times in nitrogen of litter-mates were measured before fasting and after 3, 6 and 12 hr fasting.

After 12 hr fasting rats had a lower concentration of carbohydrate in their livers than fed rats of the same age. During fasting the concentration of carbohydrate in the liver decreased rapidly, while the concentration of carbohydrate in the heart fell more slowly. After 3, 6 and 12 hr fasting, the percentage reductions in survival time and in heart carbohydrate (CHO) were closely similar; the percentage fall in liver carbohydrate was much greater (Fig. 5).

The terminal concentration of carbohydrate in the hearts (samples 15 sec after the last breath in nitrogen) of fasting and fed rats were not significantly different (Table 2), although terminal concentrations of lactic acid in the hearts and blood were lower in the fasting than in the fed rats. These results suggest that the high terminal concentration of lactic acid in the heart and blood do not prevent the utilization of the 5–6 mg/g of carbohydrate which remained in the heart when breathing stopped.

Recovery of carbohydrate stores in heart and liver and of survival times in nitrogen after a non-fatal period of anoxia

Rats from six litters (16–20 hr old) were placed in nitrogen at 36° C for about 25 min, and were then placed in air at 33° C. All the rats were breathing normally within a few minutes and appeared to recover completely. After 1 hr in air, five–six rats were decapitated, and the concentrations of blood lactic acid and of carbohydrate in the hearts and livers were measured; five–six of their litter-mates were again placed in nitrogen and the time to last breath measured. Similar observations were made after 2, 3 and 4 hr recovery in air. The results are shown in Fig. 6. After depletion by anoxia the concentration of heart carbohydrate returned almost

to its initial level in 3 hr. The time to last breath of animals exposed for a second time to nitrogen increased in the same proportion as the increase in concentration of the heart carbohydrate. The concentration of carbohydrate in the liver remained low during the 4 hr after anoxia, although the blood lactic acid fell from a mean of 148 mg/100 ml. to normal levels within 2 hr (Table 3).

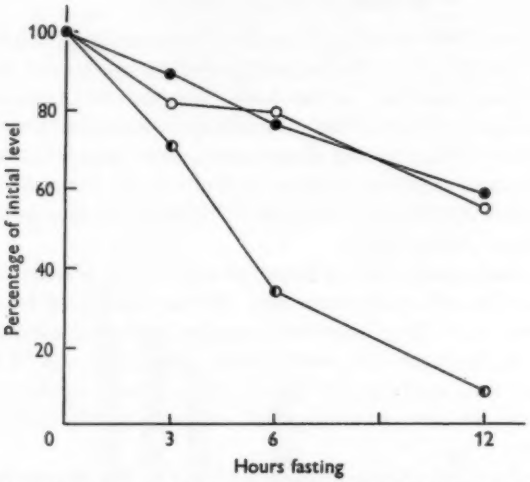


Fig. 5. Percentage changes in the concentrations of carbohydrate in the livers and hearts of new-born rats fasting for 3, 6 and 12 hr and percentage change in survival time in nitrogen of their litter-mates. Each point is the mean of fifteen observations (◑, liver; ●, heart; ○, survival time).

TABLE 2. Terminal concentrations of carbohydrate in the heart and of lactic acid in the heart and blood after exposure of new-born rats and their fasting litter-mates to nitrogen. (Each figure is the mean of 13 observations).

	Unfasted	Fasted	Significance of difference
Heart total carbohydrate (mg/g)	4·8 ± 0·5	6·3 ± 1·2	$P > 0{\cdot}1$
Heart lactic acid (mg/g)	4·2 ± 0·1	2·7 ± 0·3	$P < 0{\cdot}001$
Blood lactic acid (mg/100 ml.)	240 ± 12	152 ± 13	$P < 0{\cdot}001$

The effects of insulin on blood glucose, heart carbohydrate concentration and time to last breath in nitrogen

Figure 7 shows that insulin (1 or 2 u./g subcutaneously) caused a fall in blood glucose in new-born rats which was maximal within 2 hr. At this time after injection there was a small but significant decrease in the mean concentration of carbohydrate of the hearts from 26·4 ± 0·67 mg/g to 23·3 ± 1·1 mg/g (Table 4).

In order to test the effect of insulin on the time to last breath in nitrogen, rats from two litters less than 24 hr old were divided into two groups. Rats of one group were injected subcutaneously with 1 u./g of insulin; the others were injected with the same volume of 0·9 % NaCl. Between 1 and 2 hr after injection, all the rats were placed in nitrogen.

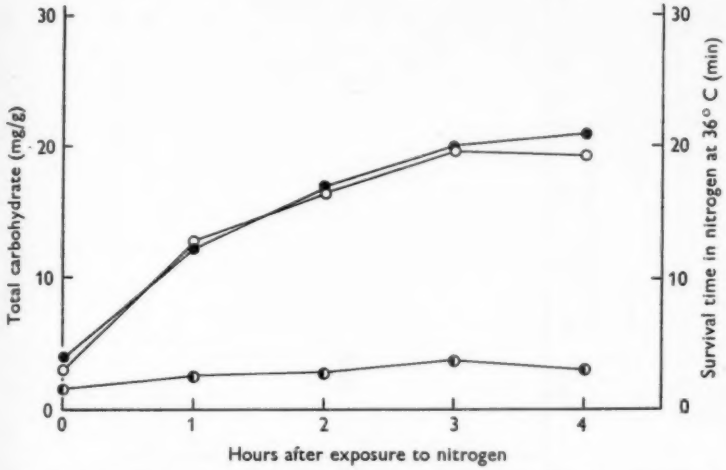


Fig. 6. Concentrations of carbohydrate in the heart and liver of new-born rats and survival times in nitrogen of litter-mates during recovery from exposure to nitrogen (each point is the mean of five or six observations; ●, heart; ◑, liver; ○, survival time).

TABLE 3. Decrease in blood lactic acid in new-born rats after a non-fatal period of anoxia. (Each estimation from 1 rat)

Hours after exposure to nitrogen	0	1	2	3
Blood lactic acid (mg/100 ml.)	137	40	12	12
	146	17	16	3
	160	68	7	9

Heart rates were recorded and the times to last breath and to last heart beat were observed. The time to last breath was decreased in insulin-treated animals. Insulin-treated animals stopped breathing after $14{\cdot}2 \pm 1{\cdot}0$ min in nitrogen, whereas litter-mate controls continued to breathe for $24{\cdot}2 \pm 1{\cdot}1$ min in nitrogen (Table 4). The mean heart rate in the control rats at 36° C before exposure to nitrogen was 316 beats/min and in the insulin-treated rats was 292 beats/min, and changes in the heart rate during exposure to nitrogen were similar in insulin-treated and control animals. The heart rate of both groups decreased rapidly during the first 4 min and then more slowly (Fig 8) and the times for which the heart con-

tinued to beat were not significantly different in the two groups of rats. From this it appears that the heart rate during exposure to nitrogen is not affected by low blood glucose levels. However, rats treated with insulin stopped breathing while the heart rate was 95 beats/min, whereas control rats stopped breathing when the heart rate was 62 beats/min.

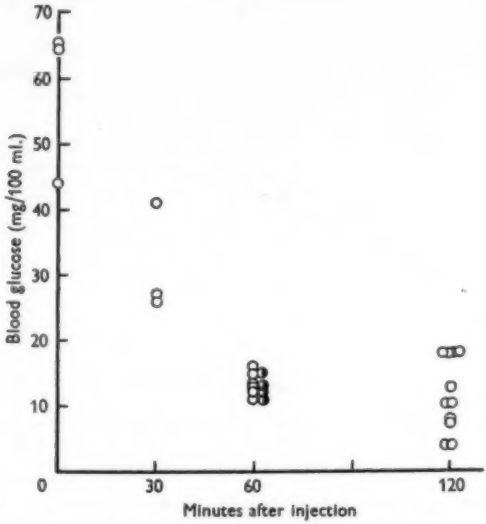


Fig. 7. Concentrations of blood glucose in new-born rats after subcutaneous injection of 1 u./g (○) or 2 u./g (◑) of insulin.

TABLE 4. The effect of insulin on carbohydrate levels in new-born rats and on their survival in nitrogen

	Initial heart carbohydrate (mg/g)	Initial blood glucose (mg/100 ml.)	Time to last heart beat in nitrogen (min)	Time to last breath in nitrogen (min)
Untreated rats	$26{\cdot}4 \pm 0{\cdot}67$ (7)	$60 \pm 3{\cdot}0$ (10)	$33 \pm 2{\cdot}6$ (7)	$24{\cdot}2 \pm 1{\cdot}1$ (7)
Insulin-treated rats (1 u./g)	$23{\cdot}3 \pm 1{\cdot}1$ (8)	$12 \pm 1{\cdot}4$ (11)	$31 \pm 1{\cdot}8$ (6)	$14{\cdot}2 \pm 1{\cdot}0$ (6)
Significance of difference between control and treated rats	$0{\cdot}05 > P > 0{\cdot}02$	$P < 0{\cdot}001$	$0{\cdot}7 < P < 0{\cdot}8$	$P < 0{\cdot}001$

(Figures in brackets indicate the number of observations).

The effects of glucose on the survival times of young rats

In the following experiments litter-mates were paired for size. Ten minutes before exposing them to nitrogen one rat of each pair was injected intraperitoneally with 0·01 ml./g of 20% glucose and the other with 0·9% NaCl.

Rats less than 24 hr old. In one litter (A) in which the blood glucose of the control rats at the time of last breath in nitrogen was greater than 97 mg/100 ml., the survival time was not increased by injection of glucose, although the blood glucose concentration was significantly increased (Table 5). In another litter (B) in which the control rats died in nitrogen

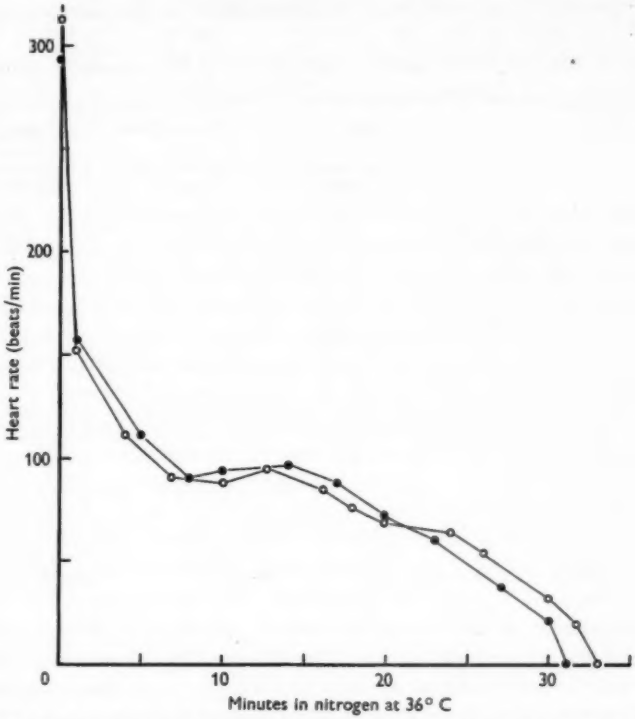


Fig. 8. The heart rates of untreated and insulin treated new-born rats during exposure to nitrogen (each point is the mean of six to seven observations; ●, insulin-treated rats, 1 u./g s.c.; ○, control rats 0·9 % NaCl.)

with a low blood glucose (< 25 mg/100 ml.) injection of glucose before exposure to nitrogen increased the survival time (Table 5).

However, in each litter there was no significant difference between the final concentrations of carbohydrate in the hearts of glucose-treated and of control rats (Table 5).

These results suggested that intraperitoneal glucose prolongs the survival time of new-born rats if the blood glucose cannot be maintained. Other conditions in which injection of glucose may be expected to prolong

the anoxic survival time were then investigated. Rats from three litters were exposed to nitrogen for 22 min and then allowed to recover in air. After 1 hr litter-mates were treated with intraperitoneal glucose or saline and 10 min later were re-exposed to nitrogen. Survival times and terminal blood glucose were measured, and under these conditions a significant prolongation of the time to last breath was found (Table 6). However, part of this increase in survival time is attributable to the increase in total

TABLE 5. Effect of glucose injection on the survival of new-born rats in nitrogen. (Each figure is the mean of 4 observations)

		Survival time in nitrogen (min)	Terminal heart carbohydrate (mg/g)	Terminal blood glucose (mg/100 ml.)
Litter A	Control	24·4	8·5	131
	Glucose-treated	24·8	9·1	223
	Significance of difference	$0{\cdot}6 < P < 0{\cdot}7$	$0{\cdot}8 < P < 0{\cdot}9$	$0{\cdot}05 < P < 0{\cdot}1$
Litter B	Control	22·8	1·5	14
	Glucose-treated	31·8	2·0	95
	Significance of difference	$P < 0{\cdot}001$	$0{\cdot}5 < P < 0{\cdot}6$	$P = 0{\cdot}02$

TABLE 6. Effect of glucose injection on the survival time in nitrogen and terminal blood glucose of new-born rats in which carbohydrate reserves were partially depleted by a previous period of anoxia. (Each figure is the mean of 11 observations)

	Mean survival time in nitrogen (min)	Terminal blood glucose (mg/100 ml.)
Controls	$17{\cdot}1 \pm 0{\cdot}9$	$10 \pm 1{\cdot}0$
Glucose-treated (2 mg/g I.P.)	$28{\cdot}4 \pm 0{\cdot}9$	95 ± 14

heart carbohydrate which occurs during the 10 min after glucose injection. For example, control rats 1 hr after the first exposure to nitrogen had a heart carbohydrate of $15{\cdot}8 \pm 1{\cdot}54$ mg/g, and their litter-mates 10 min after the injection of glucose had a heart carbohydrate of $18{\cdot}4 \pm 0{\cdot}39$ mg/g.

Rats of 4, 8 *and* 18 *days of age.* Glucose (2 mg/g in solution) was given intraperitoneally to one of a pair of litter-mates and saline intraperitoneally to the other. Ten minutes later both rats were exposed to nitrogen. The results are shown in Table 7. In rats of all ages the terminal blood glucose concentration was significantly increased by injection of glucose before exposure to nitrogen. In rats of 8 and 18 days of age, the survival time in nitrogen of glucose-injected rats was significantly increased ($P < 0{\cdot}01$), but there was no significant increase of survival time in rats 4 days old. Terminal concentrations of heart carbohydrate were not significantly different in control and glucose-treated rats.

TABLE 7. Effect of glucose injection on the survival of young rats in nitrogen: paired litter-mates were used

	Control			Glucose-treated		
Age in days	Survival time (min)	Terminal heart carbohydrate (mg/g)	Terminal blood glucose (mg/100 ml.)	Survival time (min)	Terminal heart carbohydrate (mg/g)	Terminal blood glucose (mg/100 ml.)
4	18·6 (7)	3·6 (7)	78* (7)	21·8 (7)	4·3 (6)	185* (7)
8	8·3 (8)*	0·8 (4)	134* (4)	11·1* (8)	0·9 (4)	316* (4)
18	1·6 (6)*	0·8 (6)	71* (6)	2·4* (6)	0·9 (5)	151* (6)

* Differences between control and glucose-treated groups are significant ($P < 0.01$). Figures in brackets indicate the number of observations.

DISCUSSION

During the exposure of new-born rats to nitrogen, the concentrations of carbohydrate in the heart and liver were reduced much more than those in the lung, muscle or skin. Hicks (1953) showed by histological methods that the heart and liver glycogen stores were depleted under these conditions, but the glycogen contents of the skeletal muscle and skin were not appreciably reduced. He showed that brain glycogen was low and was unable to measure histologically any change during anoxia. Shapiro & Wertheimer (1943) found that there was no phosphorylase in the skeletal muscles of the new-born rat. They also found that extracts of brains of new-born rats, because of the absence of phosphoglucomutase, did not break down glycogen. It seems therefore unlikely that endogenous glycogen can be metabolized in either the skeletal muscle or brain of new-born rats, and that blood glucose must provide most of the energy for both muscle and brain metabolism. When the rat is placed in nitrogen the availability of blood glucose to the brain and muscle must become an important factor in determining the activity of these tissues. When a new-born rat is placed in nitrogen there is a rise in blood glucose, but in a rat about 24 hr old there is no rise in blood glucose during anoxia, probably because by this age the initial liver carbohydrate has fallen too low to maintain the blood glucose level. However, as there is only a small decrease in survival time during the first 24 hr of life, it appears that blood glucose is not an important limiting factor in determining the anoxic survival. Also, injections of glucose before exposure to nitrogen produced only slight, if any, prolongation of the survival time. These results immediately cast doubt on the importance of liver glycogen reserves in the anoxic new-born rat, as liver glycogen can be made available to other tissues only after mobilization to blood glucose. We find no relation between the liver carbohydrate concentration and the time for which young rats continue to breathe in nitrogen. The most striking fall in liver carbohydrate which

was found occurred in the first 18 hr after birth and was not accompanied by a proportionate decrease in the survival time in nitrogen. Britton & Kline (1945) appear to have over-emphasized the part played by liver glycogen in determining anaerobic survival; they state: 'the relatively large size of the liver and its high concentration of glycogen (6–8%) in young rats (Corey, 1935) would, for example, appear to be involved in the striking degree of tolerance (to anoxia) observed...when liver glycogen levels have declined to approximately adult values, at 3–4 weeks of age (Britton & Silvette, 1932), anoxic resistance significantly reaches adult or even subadult levels'. We have found in normal new-born rats that neither liver glycogen nor blood glucose determines the survival in nitrogen. However, under some experimental conditions blood glucose may become a limiting factor. For example, when insulin-treated rats in which the initial blood glucose is less than 18 mg/100 ml. are exposed to nitrogen, gasping movements persisted only 14 min as opposed to 24 min in their litter-mate controls. Glucose has been found to restore to normal the survival time in nitrogen of insulin-treated rats (Himwich, Fazekas & Homburger, 1943). Glucose injection also prolongs the survival time in nitrogen of rats which have been allowed to recover partially from a previous period of anoxia.

The importance of carbohydrate in the heart. Under all the experimental conditions tested, with the single exception of the insulin-treated rats, the time to last gasp in nitrogen was proportional to the initial level of carbohydrate in the heart. First, during fasting, the survival time decreased in the same proportion as heart carbohydrate; liver carbohydrate decreased more rapidly. Secondly, during recovery from non-fatal exposure to nitrogen, heart carbohydrate and survival time increased together while liver carbohydrate remained low. Thirdly, with increasing age, survival time followed closely changes in the heart carbohydrate, but not changes in liver carbohydrate. This supports the suggestion of Dawes *et al.* (1959) that the heart carbohydrate concentration and the survival in anoxia may be causally connected. In this case, cessation of respiration would be due to failure of the circulation to the brain and respiratory muscles and their nerves. Even in insulin-treated animals, there was a correlation between the initial level of carbohydrate in the heart and the time for which the heart continued to beat in nitrogen. Only after insulin did the respiration of an anoxic new-born rat appear to fail from lack of blood glucose while there was still a relatively high heart rate.

It should, however, be emphasized that these investigations, while they support the view that the initial heart carbohydrate concentration is one factor in determining survival during anoxia, do not exclude the participation of other variables. The importance of environmental temperature

has already been referred to (Fig. 1) and has been investigated by other workers. The fall in blood pH which occurs during anoxia is another determining factor under certain experimental conditions (Dawes, Mott & Stafford, unpublished). There is a further variable related to changes in the tissues with increasing age, irrespective of the integrity of the circulation. Thus it is well known, and we have confirmed the observation, that the decapitated head of a new-born rat will continue to make respiratory efforts for many minutes, far longer than that of an adult yet less than that of an intact anoxic new-born rat. In addition, Whittam (1960) has shown that kidney slices from immature animals retain potassium during anoxia for longer than those from mature animals of the same species. In the future we shall have to study not only the decreasing ability of individual tissues to withstand anoxia with increasing age, and the reason for the fall in the carbohydrate reserves of the heart, but also the way in which these, and other factors, contribute to the astonishing ability of immature animals to withstand anoxia.

SUMMARY

1. The times to last breath of new-born rats in nitrogen increased with decreasing environmental temperature. Heart carbohydrate was depleted more rapidly at higher temperatures.

2. During exposure of new-born rats to nitrogen, the heart carbohydrate was almost depleted, the liver carbohydrate was depleted or reduced, and skin, muscle and lung carbohydrate concentrations were only slightly decreased. Increase in blood glucose was related to the concentration of carbohydrate in the liver. Loss in liver and heart carbohydrate during exposure of new-born rats to nitrogen accounted for 72% of the lactic acid production, but during the first day of life while the liver carbohydrate was rapidly falling, the time to last breath in nitrogen decreased only slightly.

3. Heart carbohydrate and survival time in nitrogen decreased steadily between 0 and 16 days of age; liver carbohydrate fell from a mean of 58 mg/g to a minimum (< 10 mg/g) during the first 24 hr of life and increased gradually to adult levels (33 mg/g) at about 16 days of age.

4. During depletion of liver and heart carbohydrate by fasting, survival time decreased in the same proportion as heart carbohydrate. When the tissue carbohydrate was reduced by exposure to nitrogen, heart carbohydrate and survival time in rats re-exposed to nitrogen recovered in the same proportion.

5. Insulin (1–2 u./g) reduced the blood glucose of new-born rats to less than 20 mg/100 ml. and reduced the mean time to last breath in nitrogen

to 14·2 min; initial heart carbohydrate and the time for which heart beats persisted in nitrogen were only slightly reduced by insulin.

6. The survival times of new-born rats in nitrogen were prolonged by intraperitoneal injection of glucose only under the following conditions: (*a*) In litters in which untreated rats died with a very low blood glucose or (*b*) in rats whose carbohydrate levels were depleted by previous exposure to nitrogen. The survival times of 8- and 18-day-old rats were slightly but significantly increased by glucose injection 10 min before exposure to nitrogen.

We are grateful to Dr G. S. Dawes for advice and encouragement throughout this work.

REFERENCES

BARKER, S. B. & SUMMERSON, W. H. (1941). The colorimetric determination of lactic acid in biological material. *J. biol. Chem.* **138**, 535–554.

BRITTON, S. W. & KLINE, R. F. (1945). Age, sex, carbohydrate, adrenal cortex and other factors in anoxia. *Amer. J. Physiol.* **145**, 190–202.

BRITTON, S. W. & SILVETTE, H. (1932). Effects of cortico-adrenal extract on carbohydrate metabolism in normal animals. *Amer. J. Physiol.* **100**, 693–700.

CHESLER, A. & HIMWICH, H. E. (1944). Comparative studies of the rates of oxidation and glycolysis in the cerebral cortex and brain stem of the rat. *Amer. J. Physiol.* **141**, 513–517.

COREY, E. L. (1935). Growth and glycogen content of the fetal liver and placenta. *Amer. J. Physiol.* **112**, 263–267.

DAWES, G. S., MOTT, J. C. & SHELLEY, H. J. (1959). The importance of cardiac glycogen for the maintenance of life in foetal lambs and new-born animals during anoxia. *J. Physiol.* **146**, 516–538.

HICKS, S. P. (1953). Developmental brain metabolism. Effects of cortisone, anoxia, fluoroacetate, radiation, insulin, and other inhibitors on the embryo, newborn and adult. *Arch. Path.* (*Lab. Med.*) **55**, 302–327.

HIMWICH, H. E., BERNSTEIN, A. O., HERRLICH, H., CHESLER, A. & FAZEKAS, J. F. (1942). Mechanism for the maintenance of life in the newborn during anoxia. *Amer. J. Physiol.* **135**, 387–391.

HIMWICH, H. E., FAZEKAS, J. F. & HOMBURGER, E. (1943). Effect of hypoglycemia and anoxia on the survival period of infant and adult rats and cats. *Endocrinology*, **33**, 96–101.

HUGGETT, A. ST G. & NIXON, D. A. (1957). Use of glucose oxidase, peroxidase, and *o*-dianisidine in determination of blood and urinary glucose. *Lancet*, **273**, 368–370.

KEMP, A. & KITS VAN HEIJNINGEN, A. J. M. (1954). A colorimetric micro-method for the determination of glycogen in tissues. *Biochem. J.* **56**, 646–648.

MENDEL, B., KEMP, A. & MYERS, D. K. (1954). A colorimetric micro-method for the determination of glucose. *Biochem. J.* **56**, 639–646.

SHAPIRO, B. & WERTHEIMER, E. (1943). Phosphorolysis and synthesis of glycogen in animal tissues. *Biochem. J.* **37**, 397–403.

WHITTAM, R. (1960). Glycolysis and potassium transport in kidney slices from new-born animals. *J. Physiol.* (in the Press).

J. Physiol. (1960), **153**, *pp.* 473–480
With 3 text-figures
Printed in Great Britain

ACTIONS OF PURE BRADYKININ

By D. F. ELLIOTT, E. W. HORTON and G. P. LEWIS
From the National Institute for Medical Research, Mill Hill, London, N.W. 7

(*Received* 9 *May* 1960)

Bradykinin, first described by Rocha e Silva, Beraldo & Rosenfeld (1949), is one of a group of polypeptides called plasma kinins. Plasma kinins are known to stimulate certain types of smooth muscle, to cause vasodilatation, to increase capillary permeability and to produce pain when brought into contact with pain fibres. It has not been clear hitherto whether bradykinin is responsible for all these actions or whether several peptides are involved, as in all experiments crude extracts have been employed. However, recently bradykinin has been isolated (Elliott, Lewis & Horton, 1960*a*) and its structure determined (Elliott, Lewis & Horton, 1960*b*,*c*). It is therefore now possible to examine the actions of the pure peptide.

The present investigation was carried out to answer the question, does the crude preparation of bradykinin contain one peptide having all the actions reported for the crude mixture, or does it contain a group of related peptides each of which is responsible for one or more of the biological actions? The results show that pure bradykinin possesses all the actions of the crude mixture, that is, smooth-muscle stimulation, vasodilatation, increased capillary permeability and pain production.

METHODS

Where comparisons have been made on a molar basis the molecular weight of bradykinin has been taken as 1131, which is the molecular weight of bradykinin dihydrochloride.

Actions on isolated smooth-muscle preparations

Guinea-pig ileum. A 3–4 cm segment of terminal ileum from animals weighing 250–400 g was suspended in a 15 ml. bath containing oxygenated Tyrode solution at 34° C. Tests were made every 4 min, and the contact time was 45 sec.

Rat uterus. Virgin rats weighing 120–200 g were injected subcutaneously with stilboestrol (10 μg/100 g) 16–18 hr before use. A 2 cm segment of uterine horn was suspended in a 10 ml. bath containing de Jalon's uterus solution at 30° C. Tests were made every 4 min and the time of contact was 45 sec.

Rat duodenum. The proximal 3 cm of duodenum from rats weighing 120–200 g was suspended in a 10 ml. bath containing de Jalon's solution at 30° C. Tests were made every 3 min, and the contact time was 30 sec.

Rabbit duodenum. The proximal 4–5 cm of duodenum from rabbits was suspended in a 15 ml. bath containing Locke's solution at 37° C. Tests were made every 3 min, and the contact time was 1 min.

Blood pressure experiments

Cats were anaesthetized with pentobarbitone sodium 40 mg/kg. The trachea was cannulated and blood pressure was recorded from the femoral artery. Intravenous injections were made into a cannula tied into the femoral vein.

Rats (300–400 g) were anaesthetized with urethane 175 mg/100 g, and were prepared by the method of Crawford & Outschoorn (1951). Blood pressure was recorded from the carotid artery with a Condon capillary manometer.

Vasodilatation was examined in the skin of the hind limb supplied by the saphenous artery. Cats were anaesthetized with chloralose 60 mg/kg. The preparation was the same as that described previously (Lewis, 1958).

Capillary permeability was examined by the method of Miles & Miles (1952). Guinea-pigs and rabbits were injected intravenously with Pontamine sky blue 60 mg/kg. A few minutes after injection of the dye pure or crude bradykinin or histamine in 0·1 ml. saline was injected intradermally into the abdominal skin. One hour later the area of blueing was measured.

Pain production was examined by the method of Armstrong, Dry, Keele & Markham (1953). Blisters were raised on the flexor surface of the forearms of human subjects by the application of cantharidin plasters, 2 × 2 cm. The plasters were applied in the evening before the experiment and allowed to act for 6 hr. The area was covered with a sterile dressing and a blister allowed to form during the night. The skin of the blister was cut away and the blister base was washed with a warm Ringer's solution containing (g/l.) NaCl 9·2, KCl 0·4, $CaCl_2$ 0·24 and $NaHCO_3$ 0·15. Drugs to be tested were dissolved in physiological saline and kept at 37° C during the experiment.

The test was carried out as follows. The solution to be tested was applied to the blister area with a Pasteur pipette until the area was filled. Each dose was allowed to act until the pain reached a plateau, or began to subside, up to a maximum of 2 min. The area was then thoroughly washed with the Ringer's solution. Doses were given at intervals of 10 or 20 or 30 min or 1 hr, and before each application the area was washed periodically with Ringer's solution. The subject was not told the nature of the applied solution; he assessed the pain intensity of each solution subjectively, grading it from 0 to + + +.

Guinea-pig bronchoconstriction

The method described by Konzett & Rössler (1940) was used. Injections were made intravenously through a cannula in the superior vena cava.

RESULTS

A comparison was made of the actions of pure bradykinin (P_6) and of the crude mixture (P_2) produced at the first stage of purification in the procedure of Elliott *et al.* (1960*a*). It was found that the increase in activity from P_2 to P_6 was of the same order when estimated on various isolated smooth-muscle preparations as well as when estimated for bronchoconstriction in guinea-pigs, for vasodilatation on the skin vessels of the cat hind limb, for depressor activity in cats and rats, for increase in capillary permeability in guinea-pigs and rabbits and for pain production in man.

Table 1 shows the relative potencies P_6:P_2 on these biological preparations. The variations shown in the Table are probably within experimental variations of the tests and suggest that all the actions of crude mixture are inherent properties of the pure peptide.

Actions on smooth muscle

Several isolated smooth-muscle preparations respond to low concentrations of pure bradykinin. The guinea-pig ileum responds with a slow contraction to bradykinin 10^{-9} and the rat uterus to 10^{-10}. It has been shown previously that crude bradykinin has an inhibitory effect on certain types of smooth muscle, particularly those showing rhythmical activity and a high resting tone. The pure peptide also has this inhibitory action. On the isolated rat duodenum bradykinin 8×10^{-9} caused only relaxation (Fig. 1), whereas with the rabbit duodenum and the rat colon the response was biphasic, an initial inhibitory action followed by a contraction.

TABLE 1. Ratios of the relative potency of pure (P_6) and crude (P_2) bradykinins in different tests

Vasodilatation	Cat skin	365
Depressor action	Cat B P.	480
	Rat B P.	265
Increased capillary permeability	Guinea-pig skin	390
	Rabbit skin	270
Smooth-muscle contraction	Guinea-pig ileum	450
	Rat uterus	405
Smooth-muscle relaxation	Rat duodenum	225
Bronchoconstriction	Guinea-pig	400
Pain production	Human blister base	180

Collier, Holgate, Schachter & Shorley (1959) have shown that crude kinins cause bronchoconstriction in anaesthetized guinea-pigs. In the present experiments pure bradykinin 0·5 μg injected intravenously into guinea-pigs caused a bronchoconstriction equal to that caused by the same dose of histamine. On a molar basis, therefore, bradykinin is about 10 times more active than histamine when tested in this way. When injections of bradykinin were made at 5 or 10 min intervals the preparation quickly lost its sensitivity to bradykinin. An interval of about 15–20 min between injections of bradykinin was required in order to avoid loss of sensitivity in this preparation.

Vasodilatation

The vasodilator action of crude plasma kinin has been studied in investigations on skin (Lewis, 1958), skeletal muscle and salivary glands (Hilton & Lewis, 1955). In the present experiments a dose as low as 100 ng of pure bradykinin caused vasodilatation in the skin of the cat hind limb compared with 10–20 ng acetylcholine. Figure 2 illustrates the vasodilatation produced by the pure peptide (P_6) compared with that produced by much larger doses of crude (P_2) bradykinin. Therefore, when calculated on a molar basis, bradykinin is approximately as active as acetylcholine on this preparation. When compared with histamine on a molar basis bradykinin is about 10 times more active as a vasodilator.

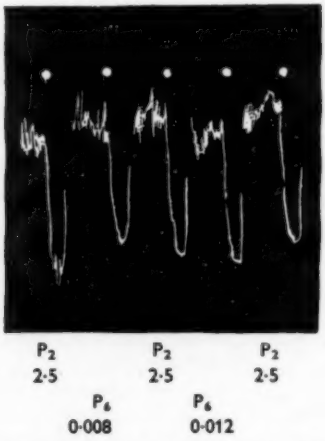


Fig. 1. Isolated rat duodenum suspended in a 10 ml. bath containing oxygenated de Jalon's solution at 30° C. Responses to crude (P_2) and pure (P_6) bradykinin; doses in μg.

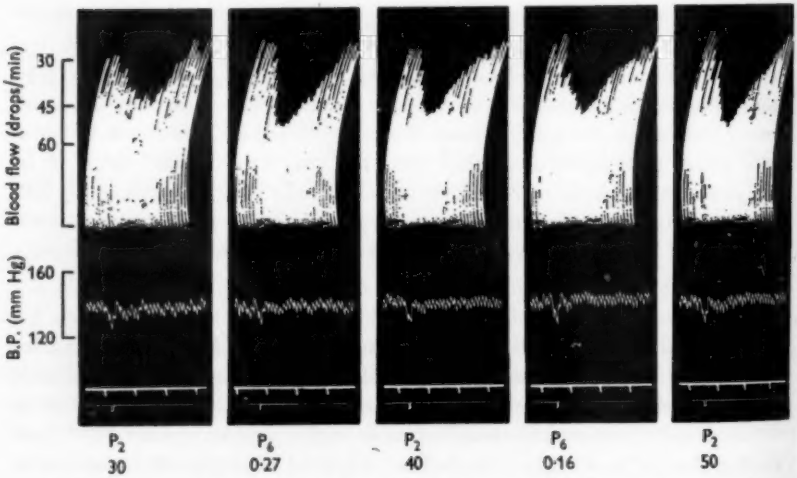


Fig. 2. Venous outflow from skin of hind limb (upper tracing) and arterial blood pressure (lower tracing). Cat, 2·5 kg, sodium pentobarbitone anaesthesia (40 mg/kg.). Intra-arterial injections of P_2 and P_6; doses in μg. Time marker, 30 sec.

Depressor action

Crude plasma kinin has been shown to lower the arterial blood pressure on intravenous injection (Rocha e Silva *et al.* 1949). Pure bradykinin injected intravenously into both anaesthetized cats and rats also caused a fall of arterial blood pressure. The threshold dose in the cat was 400 ng/kg. On a molar basis bradykinin was 10 times less active than acetylcholine and as active as histamine. The threshold dose in the rat was 150 ng/g.

Increased capillary permeability

The action of increasing capillary permeability was demonstrated for crude kinins by Holdstock, Mathias & Schachter (1957) and Herxheimer & Schachter (1959). In the present experiments intradermal injection of the pure peptide into guinea-pigs and rabbits, previously treated with Pontamine sky blue, caused an intense local blueing, indicative of increased capillary permeability (Fig. 3). In both species pure bradykinin increased capillary permeability in concentrations of 10^{-9} to 10^{-8} and therefore on a molar basis bradykinin was approximately 15 times more active in increasing permeability than histamine. Accurate comparison was difficult owing to a qualitative difference between the responses to bradykinin and those to histamine. This difference was particularly pronounced in the rabbit, where histamine produced a ring of blueing surrounding a central area which had been only slightly blued. Bradykinin on the other hand gave rise to an area of intense blueing. Furthermore, with higher concentrations of histamine (10^{-4}) the area of the response was much greater than that produced by the highest concentrations (10^{-5}) of bradykinin tested.

Stimulation of pain fibres

Armstrong, Jepson, Keele & Stewart (1957) showed that crude plasma kinin caused pain when applied to the exposed base of a cantharidin blister. In addition they claimed that large doses caused not only pain but also itching, as well as a flare, and a wheal (Armstrong, Keele, Jepson & Stewart, 1954). In the present experiments with pure bradykinin pain was produced when concentrations of 10^{-7} to 10^{-6} were applied to a blister base, but there was no itching, no flare and no wheal. On a molar basis pure bradykinin was somewhat less active than 5-hydroxytryptamine, which was the most potent pain-producing substance in the experiments of Armstrong *et al.* (1953; 1957). Histamine was usually active at a concentration of 10^{-6} but the sensation produced by histamine was different from that produced by bradykinin or 5-hydroxytryptamine. Histamine caused itching or pricking, whereas bradykinin and 5-hydroxytryptamine caused a burning sensation. Even when histamine 10^{-4} was

applied to the blister base no painful burning sensation resulted. With concentrations of bradykinin 10^{-7} to 10^{-6} the pain was transient; it sometimes disappeared in less than 1 min. With concentrations of 10^{-6} to 10^{-5} pain lasted for 2–3 min.

Repeated administration of bradykinin resulted in a gradual decrease in the sensitivity of the preparation, as is shown by the following experiment. Two exposed blister bases in the same subject were each tested at

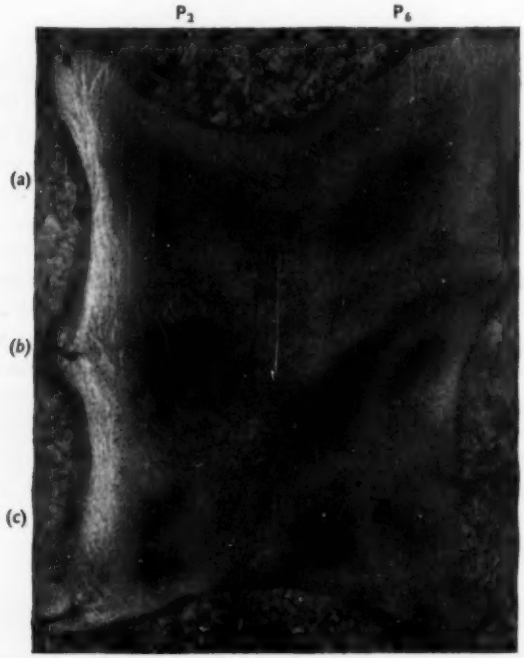


Fig. 3. Effect of intradermal injections of bradykinin on capillary permeability in a guinea-pig injected intravenously with Pontamine sky blue (60 mg/kg). The dark areas represent the areas of blueing 1 hr after the injections. Crude bradykinin (P_2) was injected in doses of (*a*) 1 mg. (*b*) 100 μg and (*c*) 10 μg. Pure bradykinin (P_6) was injected in concentrations of (*a*) 5 μg/ml., (*b*) 0·5 μg/ml. and (*c*) 0·05 μg/ml.

20 min intervals, one with bradykinin 0·1, 1·0, 1·0, 1·0 and 10·0 μg/ml. and the other with histamine 1·0 μg/ml. The application of bradykinin 0·1 μg/ml. produced a painful burning sensation but the effect of 10 times this concentration 20 min later was no greater and no longer-lasting. Subsequent applications of 1 μg, 1 μg and 10 μg/ml. produced no sensations. On the other hand, the five histamine applications to the other blister produced effects without any loss of sensitivity. After a further

20 min the blister base treated with bradykinin failed to respond even to 500 μg/ml. bradykinin, whereas the blister base which had only received histamine responded to 0·1 μg/ml. bradykinin with the usual burning sensation. Finally, both blisters, the one sensitive and the other insensitive to bradykinin, responded with a strong sensation of pain on application of 5-hydroxytryptamine creatinine sulphate 0·1 μg/ml.

DISCUSSION

All the pharmacological actions attributed to the crude plasma kinins were found to be inherent properties of the pure peptide, bradykinin. As a vasodilator substance bradykinin was found to be as active as acetylcholine, the most potent naturally occurring vasodilator substance. In increasing capillary permeability bradykinin was found to be more active than the most active substance, histamine. In producing superficial pain bradykinin was almost as active as 5-hydroxytryptamine.

Isolated smooth muscle preparations are the most sensitive tissues to bradykinin and are therefore the most commonly used in its detection. The rat uterus contracts in the presence of 0·1 ng/ml. of bradykinin, which is therefore about as active as the polypeptide oxytocin on this preparation. The rat duodenum is relaxed by bradykinin; the concentration necessary is less than the concentration of adrenaline which is required to relax it.

The action of bradykinin on the blood vessels is to dilate them and to increase their permeability—this is perhaps its most important action. This pronounced vasodilator action is consistent with the view that bradykinin plays an important role in functional vasodilatation (Hilton & Lewis, 1957). The finding that bradykinin was less active than acetylcholine in lowering the blood pressure, in spite of the fact that acetylcholine is destroyed in the blood much more rapidly, suggests that it acts on a part of the vascular tree different from that on which acetylcholine acts, and which is involved mainly in local vascular effects.

The differences we observed between bradykinin and histamine in their effects on capillary permeability were similar to those described by Menkin (1936) between histamine and a mixture of peptides called 'leukotaxine', and between histamine and crude plasma kinin described by Bhoola & Schachter (1959).

Thus bradykinin in low concentration produces two of the essential reactions of the inflammatory response—vasodilatation and increased capillary permeability. In addition it produces the typical burning sensation which is experienced in conditions of local tissue injury. Thus bradykinin may play the role of chemical mediator of the inflammatory response.

SUMMARY

1. The properties of pure bradykinin have been examined and compared quantitatively with those of a crude preparation of bradykinin.

2. The pure peptide was found to have the following actions: vasodilatation in the skin of the cat's hind limb; depressor action in cats and rats; increased capillary permeability in guinea-pigs and rabbits; contraction of isolated guinea-pig ileum and rat uterus; relaxation of rat duodenum; bronchoconstriction in guinea-pigs; and pain production when applied to the exposed base of a cantharidin blister in man.

3. It therefore seems likely that all the actions so far attributed to the crude plasma kinins can be accounted for by this single peptide, bradykinin.

REFERENCES

ARMSTRONG, D., DRY, R. M. L., KEELE, C. A. & MARKHAM, J. W. (1953). Observations on chemical excitants of cutaneous pain in man. *J. Physiol.* **120**, 326–351.

ARMSTRONG, D., KEELE, C. A., JEPSON, J. B. & STEWART, J. W. (1954). Development of pain-producing substance in human plasma. *Nature, Lond.*, **174**, 791–792.

ARMSTRONG, D., JEPSON, J. B., KEELE, C. A. & STEWART, J. W. (1957). Pain-producing substances in human inflammatory exudates and plasma. *J. Physiol.* **135**, 350–370.

BHOOLA, K. D. & SCHACHTER, M. (1959). A comparison of serum kallikrein, bradykinin and histamine on capillary permeability in the guinea-pig. *J. Physiol.* **149**, 80*P*.

COLLIER, H. O. J., HOLGATE, J. A., SCHACHTER, M. & SHORLEY, P. G. (1959). An apparent bronchoconstrictor action of bradykinin and its suppression by some anti-inflammatory agents. *J. Physiol.* **149**, 54*P*.

CRAWFORD, T. B. B. & OUTSCHOORN, A. S. (1951). The quantitative separation of adrenaline and noradrenaline in biological fluids and tissue extracts. *Brit. J. Pharmacol.* **6**, 8–20.

ELLIOTT, D. F., LEWIS, G. P. & HORTON, E. W. (1960*a*). The isolation of bradykinin: A plasma kinin from ox blood. *Biochem. J.* **74**, 15*P*.

ELLIOTT, D. F., LEWIS, G. P. & HORTON, E. W. (1960*b*). The structure of bradykinin. *Biochem. J.* (in the Press).

ELLIOTT, D. F., LEWIS, G. P. & HORTON, E. W. (1960*c*). The structure of bradykinin: a plasma kinin from ox blood. *Biochemical and Biophysical Research Communications.* New York: Academic Press.

HERXHEIMER, A. & SCHACHTER, M. (1959). Weal and flare in human skin produced by histamine and other substances. *J. Physiol.* **145**, 34*P*.

HILTON, S. M. & LEWIS, G. P. (1955). The mechanism of the functional hyperaemia in the submandibular salivary gland. *J. Physiol.* **129**, 253–271.

HILTON, S. M. & LEWIS, G. P. (1957). Functional vasodilatation in the submandibular salivary gland. *Brit. med. Bull.* **13**, 189–196.

HOLDSTOCK, D. J., MATHIAS, A. P. & SCHACHTER, M. (1957). A comparative study of kinin, kallidin and bradykinin. *Brit. J. Pharmacol.* **12**, 149–158.

KONZETT, H. & RÖSSLER, R. (1940). Versuchsanordnung zu Untersuchungen an der Bronchialmuskulatur. *Arch. exp. Path. Pharmak.* **195**, 71–74.

LEWIS, G. P. (1958). Formation of plasma kinins by plasmin. *J. Physiol.* **140**, 285–300.

MENKIN, V. (1936). Studies on inflammation. XII. Mechanism of increased capillary permeability. A critique of the histamine hypothesis. *J. exp. Med.* **64**, 485–502.

MILES, A. A. & MILES, E. M. (1952). Vascular reactions to histamine, histamine-liberator and leukotaxine in the skin of guinea-pigs. *J. Physiol.* **118**, 228–257.

ROCHA E SILVA, M., BERALDO, W. T. & ROSENFELD, G. (1949). Bradykinin, a hypotensive and smooth muscle stimulating factor released from plasma globulin by snake venoms and by trypsin. *Amer. J. Physiol.* **156**, 261–273.

J. Physiol. (1960), **153**, *pp.* 481–491
With 1 *plate and* 2 *text-figures*
Printed in Great Britain

VALVULAR INCOMPETENCE IN THE VENOUS DRAINAGE OF THE UDDER

BY J. L. LINZELL

From the Agricultural Research Council Institute of Animal Physiology, Babraham, Cambridge

(*Received* 13 *May* 1960)

This work is the basis of the following paper (Linzell, 1960) on long-term measurement of udder blood flow in conscious goats via the readily accessible caudal superficial epigastric or 'milk' vein (in front of the udder), which is the time-honoured source of mammary venous blood. The whole venous drainage of the udder has been re-examined because Linzell & Mount (1955) found valves in this vein pointing towards the udder and not away from it.

Although valves (but not their direction) are mentioned by Palić (1954) in the sheep and have been described in the cow (Becker & Arnold, 1942), the actual direction of blood flow has only been determined for the minor perineal vein joining the udder and vulva (Gracev, 1949; Gracev & Ivanov, 1955), where valves have been known for some time (Becker, 1937; Swett & Matthews, 1949). This has now been determined for all the major veins in conscious and anaesthetized goats of both sexes and various ages, and in some sheep and cattle in addition.

The results show that the 'milk' vein carries blood towards the udder in virgin females and males. In lactating females some or all of the valves are incompetent, and when the animal is standing the direction of flow is reversed. Under these conditions the 'milk' vein may carry blood from the udder, the perineum and the abdominal wall or mixtures from all three sources. Conditions are described by which mainly mammary blood may be obtained and estimates made of blood flow over long periods in conscious goats.

Of wider interest is the occurrence of valvular incompetence under physiological conditions. In multiparous females the vessels concerned have all the characteristics of varicose veins.

METHODS

Detailed dissections have been made to determine the presence of valves in the vessels of the abdominal wall in 18 sheep (Clun forest, Dorset horn and Welsh mountain), 32 goats (Saanen, British Alpine and English), 20 cattle (Jersey, Ayrshire and Shorthorn, Angus × Beef Shorthorn), 1 mare (Clydesdale) and 6 pigs. The tissues were unfixed; the majority

were examined whilst they were still warm and the blood fluid. The position of the valves could be determined by examination of the unopened veins, which were also carefully examined by the classical method of Harvey for the direction of flow and competence of the valves. In many cases fluids were injected through fine needles and many valves were tested to determine the pressures which they would withstand before allowing back flow. The veins were then completely opened longitudinally with fine Mayo scissors in the direction the valves were pointing and the valve flaps examined. Six sheep, 11 goats, 2 cattle and 2 pigs were examined under anaesthetic before dissection. The animals were supported so that they could be examined either supine, on their sides, or in a standing position. The direction of blood flow was determined by the injection through fine needles of saline, Evans Blue dye, milk, air or radio-opaque fluids.

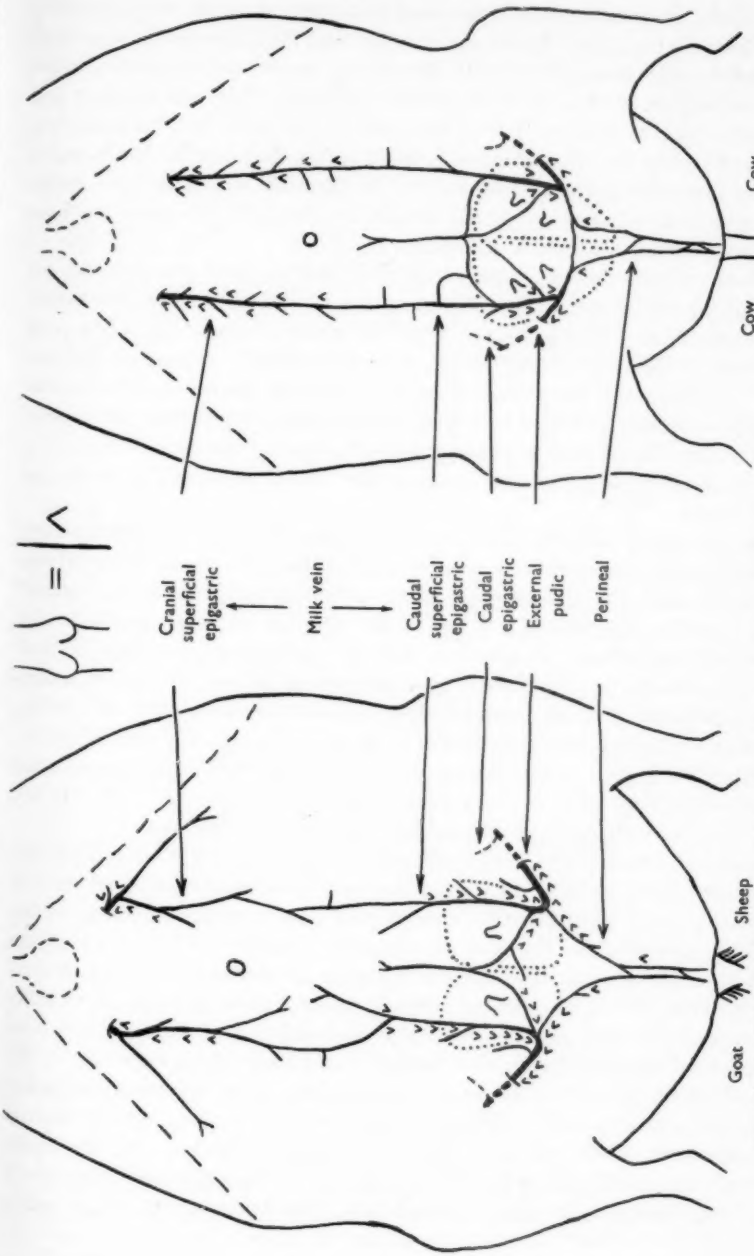
The direction of venous blood flow was also determined in some conscious animals by the use of radio-opaque fluids or isotopes (^{24}Na or ^{32}P). Goats were examined in a stand in the laboratory with which they were familiar, and cattle in their normal surroundings on the farm. In some goats repeated venepuncture was facilitated by the previous exteriorization of the caudal superficial epigastric vein as a skin-covered loop, in others and in cattle by threading polythene tubing (outside diameter 0·5 mm) into the vein.

RESULTS

The arteries and veins to the cow's udder were first described by Fürstenberg (1868) and since then they have been studied by several anatomists in all ruminants (see review by Turner, 1952). As a result it is well known that there are three main veins associated with the gland on each side: (*a*) the external pudic running from the base of the udder through the inguinal canal, (*b*) the caudal superficial epigastric vein (subcutaneous abdominal or '*milk*' vein) running from the front of the gland forward superficially along the abdomen and (*c*) the perineal, which is smaller than the other two and runs from the back of the udder to the vulva (Text-figs. 1; 2*a*, *b*).

In this work valves were found in all the main veins in the mammary region of the sheep, goat, cow, mare and pig. They were also present in the same veins in males.

Sheep and goat. The dissection of the entire abdomen of new-born and foetal animals of both sexes showed quite clearly that the skin of the ventral abdomen from the umbilicus to the perineal region is drained by the external pudic vein, which empties into the external iliac vein after passing through the inguinal canal (Text-fig. 1). The external pudic is formed by the junction of the caudal superficial epigastric and the perineal veins in the inguinal region. The perineal vein drains the skin between the vulva and the inguinal region and the caudal superficial epigastric that from the inguinal region to the umbilicus. The direction of blood flow is shown by the valves (2–17 in the goat, 2–6 in the sheep), by the angle at which the branches join the main vessels and by the fact that the vessels get smaller as they get further from their point of junction to form the external pudic vein. The perineal vein anastomoses with the veins around the anus and vulva, which are branches of the internal pudic veins within



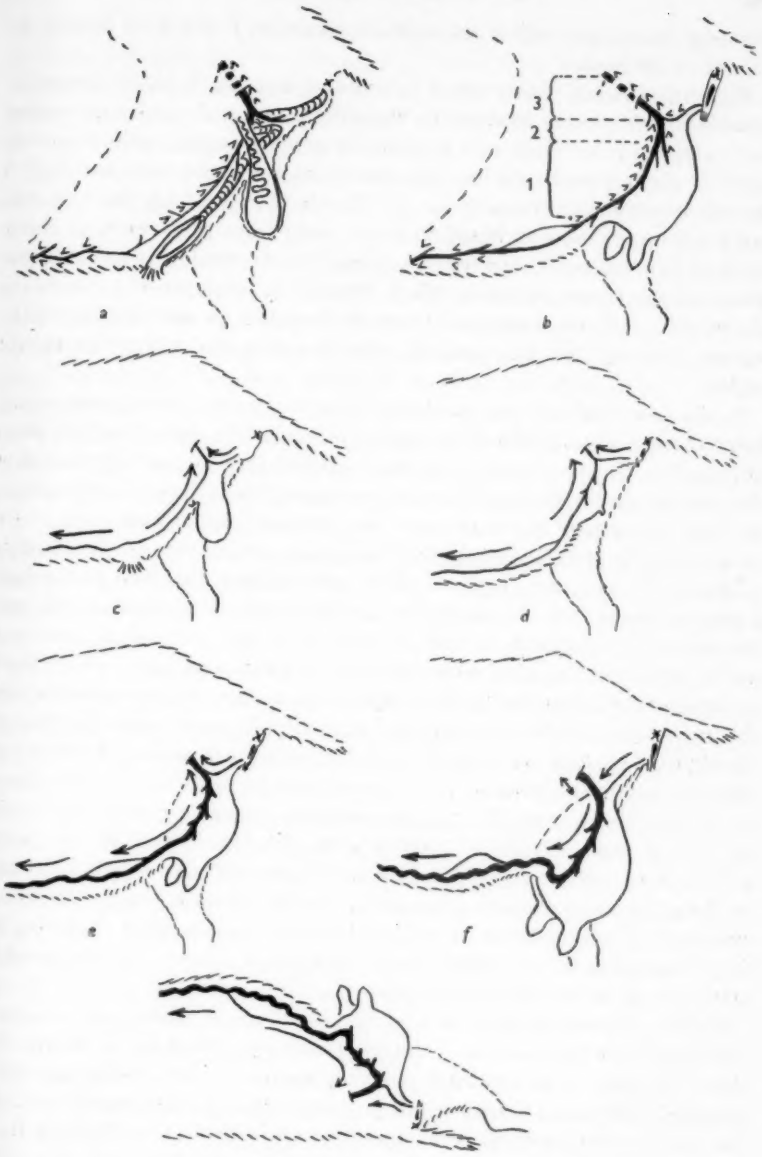
Text-fig. 1. Diagram of the superficial veins of the ventral abdomen in the goat, sheep and cow, showing the angles of branching and the average numbers of valves found. The arteries are similar.

the pelvis. In some animals there are 1 or 2 valves near the vulva directing flow towards that area. In the region of the umbilicus the caudal superficial epigastric vein anastomoses with the cranial superficial epigastric, which drains the skin in front of the umbilicus cranially. This vein enlarges as it runs forward, has valves (1–6 in the goat, 2–3 in the sheep) directing flow forward and its branches also run forward before they join it. In the region of the anastomosis the side branches join the main vessel at right angles. The valves in all these vessels in very young animals did not allow back flow even under a pressure of 250 mm Hg.

Thus, on anatomical grounds it is clear that at birth the perineal and caudal superficial epigastric veins carry blood towards the inguinal region, the opposite direction to that in which they were thought for many years to drain in the cow. Although she does not mention valves, similar conclusions about the superficial epigastric veins are drawn by Vladimirova (1954) from the size of the vessels at the anastomosis and from the angle of branching. The dissection of older but still virgin goats and sheep and of males showed that in these animals the valves were still present and competent.

In the case of females that were pregnant or lactating or had lactated, the superficial epigastric and external pudic veins were greatly enlarged concomitant with the development of the mammary glands in the inguinal region of the ruminant (Pl. 1, figs. 7–10). The valves in the perineal veins were still competent. However, in marked contrast many of the valves of the caudal superficial epigastric veins within and in front of the udder were completely incompetent or allowed back-flow at pressures of 10–40 mm Hg. In many older animals they had even atrophied and could easily be overlooked (Pl. 1, fig. 2). The valves of the external pudic veins always had better flaps than those in the caudal superficial epigastric (Pl. 1, fig. 1), but in a few old animals they allowed back-flow at low pressures.

Determination of the actual direction of blood flow in males and virgin females confirmed that all the valves were competent. The perineal and caudal superficial epigastric veins carried blood, in the direction of the valves, towards the inguinal or mammary region, and then along the external pudic veins through the inguinal canal to the abdominal vena cava. This was so in both anaesthetized and conscious animals, whether they were standing, supine or lying on their sides (Text-fig. 2). The cranial superficial epigastric vein also carried blood forward as expected in the direction of the valves. Since the cranial and caudal superficial epigastric veins anastomose in the mid-abdominal region, there is a point of reversal where some blood can go forward and some backward. In the virgin female and male this point of reversal was behind the umbilicus about midway between the last forward- and first backward-pointing valves



Text-fig. 2. Positions of valves in male goat (*a*) and female goat (*b*): (1) valves always incompetent in breeding females; (2) valves usually incompetent in older females; (3) valves sometimes partially incompetent in old females. *c–g*, usual direction of venous blood flow: *c*, males of all ages; *d*, virgin females; *e*, primiparous females; *f*, multiparous females; *g*, females of all ages, when held on the back.

(Text-fig. 2*c*, *d*), although it was shifted somewhat forward by placing the animal on its back.

The examination of blood flow in breeding females, both conscious and anaesthetized, showed that in the standing position the point of reversal had moved further back well into the mammary region, indicating that some of the valves in the caudal superficial epigastric vein had in fact become incompetent (Text-fig. 2*e*, *f*). Thus in these animals this vein does carry mammary venous blood forward away from the udder, as earlier workers have believed. However, as might be expected, this was only true when the animal was standing. When it was on its back the flow reverted to the virgin condition, backward towards the udder in the direction of the valves (Text-fig. 2*g*). The perineal vein always carried blood towards the udder.

In the sheep and goat the number of valves that were incompetent varied, but to some extent depended upon the parity of the animal and the stage of lactation. In the course of the first lactation only the anterior valves in the caudal superficial epigastric vein were incompetent in 8 out of 11 animals, so that about half the mammary venous blood went backward to the external pudic and the rest forward along the 'milk' vein in the standing position (Text-fig. 2*e*). In 9 out of 14 older animals that had had at least 2 pregnancies all the valves in the caudal superficial epigastric vein were incompetent right back to the junction with the perineal and external pudic veins. In this case, when standing, all the mammary venous blood drained forward along the 'milk' vein but, in addition, the perineal flow from the vulva went this way as well, and the external pudic carried little or no blood (Text-fig. 2*f*). In 3 multiparous non-lactating animals the flow was also reversed in the initial part of the external pudic veins, so that some or all of the blood from the caudal epigastric veins (draining the caudal abdominal muscles) flowed downwards into the superficial epigastric veins to mix with mammary venous blood, although this was not the case in the same animals during lactation. In one of these the proportion of mammary blood fell from 70–80 % of the total to only 30–50 % during the first three-fifths of pregnancy, but rose again to 70–85 % as the absolute mammary flow increased near term.

Cattle. Dissections showed that the anatomy in males and females is almost exactly the same as in the sheep and goat (Text-fig. 1) except that there are only 1–5 backward-pointing valves in the caudal superficial epigastric veins and 6–14 forward-pointing valves in the cranial vein. All the valves were found to be competent to pressures up to 250 mm Hg in male and female calves and in adult virgin heifers (2½–3 yr old). In a heifer that died during its first parturition the caudal valves were still competent to normal venous pressures; in a cow that died just before its second

parturition 2 caudal valves were incompetent and 2 competent; and in a cow that had had 2 calves all three valves were incompetent. Examination of other animals, where the exact age and parity were not known, bore out these findings that the valves tended to become incompetent with the growth of the mammary tissue, as in sheep and goats.

The direction of blood flow in the superficial epigastric veins was determined periodically in 4 animals (2 dairy and 2 beef breeds) from early calfhood onwards. Although the point of reversal up to 6–9 months of age was in front of the udder, by 12 months (well before puberty) it had moved back to about the level of the front teats where the first backward-pointing valve is found (Text-fig. 1). The valves were still competent in one of these beef heifers killed at 2 yr old (unmated), and in one of the dairy heifers (unmated) examined under anaesthesia at $2\frac{1}{2}$ yr old. It seems likely, therefore, that the cow resembles the sheep and goat.

Varicosity. In the dairy cow and dairy goat, which have been selected for abnormally large milk yields, the mammary glands are also greatly enlarged, resulting in an enormous increase in blood flow through the inguinal region. In these animals the veins are even more enlarged than those of the beef cow or sheep and in old animals are often exceedingly tortuous and thin-walled (Pl. 1, fig. 11). Compton (1953) has described three cases of fatal haemorrhage in 4 cows whose 'milk' veins developed large dilatations.

Histology. Examination of the external pudic, superficial epigastric and perineal veins and, for comparison, the femoral vein revealed differences that were affected by age. In the femoral vein, which is completely surrounded by leg muscles in ruminants, the elastic tissue was more prominent in both the adventitia and intima than the smooth muscle in the medial coat, a characteristic that became more pronounced in older animals (Pl. 1, figs. 3, 4). However, in young female and male animals the elastic and muscular tissues were about equally prominent in the three superficial 'inguinal' veins (Pl. 1, fig. 5). In primiparous animals this was still true, but in older breeding females (2 parturitions or more), the muscular coats of all three veins were much more prominent than the elastic tissue, particularly in the external pudic and superficial epigastric vessels (Pl. 1, fig. 6).

Horse and pig. The dissection of the inguinal region of a 9-yr-old Clydesdale mare that had probably not been pregnant revealed 6 valves all competent in each of 3 veins that joined to form the external pudic.

In 6 piglets there were only 1–2 valves in the caudal superficial epigastric and 3–5 in the cranial veins. All were competent and when examined under anaesthesia the flow was as would be expected from the position of the valves.

DISCUSSION

It is unnecessary to stress the importance of these findings (summarized in Text-fig. 2) for work involving blood flow measurements or mammary venous blood sampling in the cow and goat. The classical accounts of the blood vessels of the udder, which described three veins constantly draining each half, overlooked not only the valves and disposition of the branches (clearest in the young animal) but also the fact that the same vessels are present in the male, and in other mammals without inguinal mammary glands. In many non-pregnant multiparous animals, when standing, the superficial epigastric vein probably carries all the venous blood from one half of the udder, while if the flow from each half is required separately, the vessels which cross the mid line between the two halves must be removed beforehand. In pregnant multiparous animals there is the possibility that the caudal superficial epigastric veins may also carry some blood from the abdominal muscles. The fact that the blood in the perineal vein, which drains the vulval region and not the udder, may mix with the mammary venous blood must also be considered, although the amount is very small in relation to the mammary venous flow. Russian workers (Vladimirova, 1954; Gracev & Ivanov, 1955) believe that the perineal vein carries hormones in the venous blood from the genital organs direct to the mammary glands. This is extremely unlikely. There is not a true portal system breaking up separately into the capillaries of the udder and the anatomical and physiological findings described here show clearly that the vulval venous blood merely mixes with the mammary venous blood and returns with it to the general circulation.

When the animal lies down, the superficial epigastric veins are compressed on one side and then of course all mammary venous blood must flow through the external pudic veins. This conclusion has been confirmed by tying the external pudic veins in 2 multiparous goats in which the flow was all along the 'milk' vein in the standing position. In both cases numerous collaterals opened up around the tied vein and drainage through the inguinal canal was quickly re-established. Resection of the perineal veins is also followed by the growth of new vessels draining from the vulva to the external pudic vein.

In order to sample or measure total mammary venous blood flow in the conscious undisturbed goat it is necessary to know the point of reversal of blood flow in the caudal superficial epigastric or external pudic veins in the animal concerned at that particular time. This can be determined by passing a fine catheter back along the vein from just in front of the udder and radiographic observation after the injection of a radio-opaque fluid. However, thrombosis is very easily produced by this procedure in the goat and there

is the possibility that the valves are altered by the catheter. Ligation of the perineal and external pudic veins, as used by Graham (1937), is unsatisfactory because a collateral drainage is quickly established, and the operation does not ensure that the total drainage is via the caudal superficial epigastric vein, as he assumed. For short-term experiments a polythene inflatable cuff may be fixed round the external pudic vein so that it can be temporarily occluded, but this ultimately causes thrombosis with collateral growth and is thus unreliable for long-term experiments. A simpler and more repeatable procedure is to compress the external pudic vein manually between the base of the udder and the inguinal canal. This vessel lies immediately in front of the artery, which can be easily palpated. This procedure, with compression of the perineal vein (just below the vulva), ensures that all, and only, the mammary blood leaves via the superficial epigastric vein in a multiparous goat where all the valves are almost certainly partially, if not completely, incompetent. If the flow in the superficial epigastric vein is measured at the same time, compression of these veins will provide information as to the percentage of mammary venous blood leaving via the two channels open to it, and the extent to which it is diluted with blood from the vulva or abdominal wall.

The occurrence of valvular incompetence in many female goats, sheep and cows is of wider physiological interest. The increase in diameter, elongation, tortuosity, valvular incompetence, and hypertrophy of the muscular media may all be considered as responses to the increase of blood coming into the area on account of the growth of the mammary gland. King (1950) has emphasized the frequency in the human of varicose veins in the legs during pregnancy and believes that oestrogenic hormones may be implicated. However, in the goat the changes occur late in the first pregnancy, after the main lobulo-alveolar growth of the mammary gland has taken place, at the time of rapid mammary enlargement and commencing milk secretion. Moreover, in animals where the valves in the external pudic veins are incompetent, the amount of abdominal blood running in the superficial epigastric vessels is greatly increased in late pregnancy. This suggests that abdominal pressure and/or pressure of the uterus or foetus on the pelvic veins is an important factor as it may well be in the human. At any event it would seem that the 'milk' veins of the female ruminant are an exception to the statement of Foote (1954) that varicose veins are only known in man, since all the above changes are those that are characteristic of that condition.

SUMMARY

1. The main veins of the ruminant udder have been re-examined anatomically and valves have been found in all of them. The actual directions of

blood flow have been determined in animals of various ages, conscious and anaesthetized.

2. In the male and virgin female the external pudic veins carry all the mammary venous blood, together with some blood from surrounding areas.

3. In breeding females the external pudic and the superficial epigastric veins are greatly enlarged, the muscular coats hypertrophied, and the valves partially or completely incompetent. When the animal is standing the flow in the caudal superficial epigastric, and sometimes the external pudic veins, is in the opposite direction to that in the male and virgin female, and some or all of the mammary venous blood leaves via the former vessel, although it may also carry some blood from the perineum and abdominal wall.

4. The sampling and measurement of mammary venous blood flow in the conscious goat is discussed in the light of these findings.

5. This is an example of varicose veins occurring under physiological conditions.

The histological sections were prepared by Mr W. S. Hardy and the photographs taken by Mr A. W. Attwood and Mr A. L. Gallup.

REFERENCES

BECKER, R. B. (1937). Certain points in the physiological processes of the cow. *J. Dairy Sci.* **20**, 408–410.

BECKER, R. B. & ARNOLD, P. T. D. (1942). Circulatory system of the cow's udder. *Bull. Flo. Agric. Exp. Sta. Tech.* p. 379.

COMPTON, L. S. (1953). Varicosis of the milk vein in dairy cattle. *Cornell Vet.* **43**, 362–365.

FOOTE, R. R. (1954). *Varicose Veins*, 2nd ed. St Louis, Missouri: C. V. Mosby.

FÜRSTENBERG, M. H. F. (1868). *Die Milchdrüsen der Kuh.* Leipzig: W. Englemann.

GRACEV, I. I. (1949). Mammary gland reflexes. *J. gen. Biol., Moscow*, **10**, 401–420.

GRACEV, I. I. & IVANOV, H. E. (1955). Blood supply to the mammary glands in the cow. *C.R. Acad. Sci. U.R.S.S.* **104**, 939–940.

GRAHAM, W. R. (1937). The utilisation of lactic acid by the lactating mammary gland. *J. biol. Chem.* **122**, 1–9.

KING, E. S. J. (1950). The genesis of varicose veins. *Aust. N. Z. J. Surg.* **20**, 126. (Cited by FOOTE, R. P., 1954.)

LINZELL, J. L. (1960). Mammary gland blood flow and oxygen, glucose and volatile fatty acid uptake in the conscious goat. *J. Physiol.* **153**, 492–509.

LINZELL, J. L. & MOUNT, L. E. (1955). Variations in the direction of venous blood flow in the mammary region of the sheep and goat. *Nature, Lond.*, **176**, 37–38.

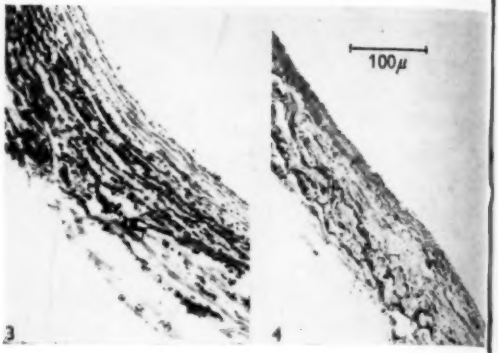
PALIĆ, D. (1954). The blood supply of the sheep's udder. *Acta Vet. Belgrade*, **4**, 85–97.

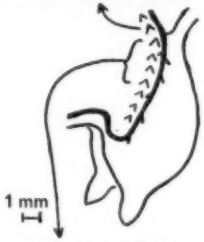
SWETT, W. W. (1941). The course of venous circulation of the udder. *Ann. Rept. Chief of the Bureau of Dairy Industry, U.S.D.A.* p. 16.

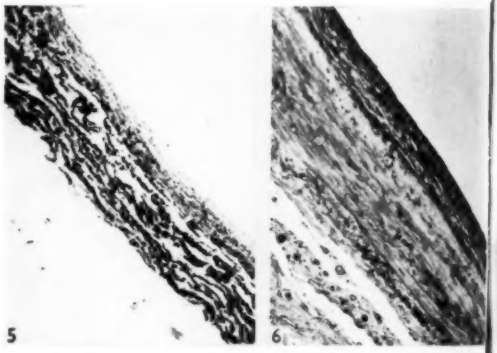
SWETT, W. W. & MATTHEWS, C. A. (1949). Some studies of the circulatory system of the cow's udder. *U.S.D.A. Tech. Bull.* 982.

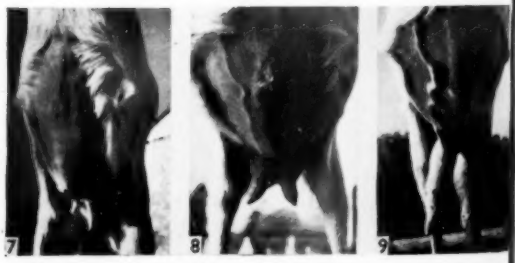
TURNER, C. W. (1952). *The mammary gland, Vol.* 1. *The anatomy of the udder of cattle and domestic animals.* Columbia, Missouri: Lucas.

VLADIMIROVA, A. D. (1954). The blood supply to the udder. *Bull. Leningrad Univ.* No. 7. 97–107.









J. L. LINZELL

(*Facing p.* 491)

EXPLANATION OF PLATE

Figs. 1, 2. Valves from 2½-year-old female goat killed during the second lactation; during life the venous flows were as shown in Text-fig. 2*f*. (1) Competent valve from external pudic vein. (2) Four incompetent valves from caudal superficial epigastric vein within the udder.

Figs. 3–6. Transverse sections of femoral (3, 4) and caudal superficial epigastric veins (5, 6) of adult male (3, 5) and female (4, 6) goats, fixed distended and stained Verhoeff Van Gieson (elastic tissue black). In the male the veins are not significantly different but in the female the muscular coat of the caudal superficial epigastric vein is hypertrophied and there is little elastic tissue.

Figs. 7–10. The superficial epigastric veins in living Saanen goats: 7, virgin female at puberty, 8 months old; 8, female at first parturition, 13 months old; 9, female at fifth parturition, 7 years old; 10, male 4½ years old.

Fig. 11. Unusually varicose superficial epigastric veins of adult multiparous British alpine goat. The condition was present before the veins were exteriorized for flow measurements. During lactation the flow in the vein loop was smoothly laminar but when the flow had decreased at the end of lactation eddies and stagnant pockets were found even in the loops.

J. Physiol. (1960), **153**, *pp.* 492–509
With 7 text-figures
Printed in Great Britain

MAMMARY-GLAND BLOOD FLOW AND OXYGEN, GLUCOSE AND VOLATILE FATTY ACID UPTAKE IN THE CONSCIOUS GOAT

By J. L. LINZELL

From the Agricultural Research Council Institute of Animal Physiology, Babraham, Cambridge

(*Received* 13 *May* 1960)

It has been shown that in the isolated perfused mammary gland the blood flow is often lower than that measured through the gland *in situ* in anaesthetized animals (Linzell, 1950; Hebb & Linzell, 1951). In an investigation of the secretory activity of the perfused udder of the goat (Cargill-Thompson, Drury, Hardwick, Linzell & Tucker, 1958), it was felt desirable to know the blood flow through the udder of the conscious animal in order to assess the importance of this factor in the reduced level of milk secretion that is characteristic of this preparation. Figures were also needed for O_2 consumption and for the uptake of some milk precursors.

Apart from one figure quoted by Graham (1937), which was given without the weight of the tissue, the only published figures for udder blood flow in the conscious goat are those of Jung (1932*a*, *b*, 1933) who found values of 5·5–7·2 ml./100 g/min and Vladimirova (1955) who gives 4–18·7 for conscious and anaesthetized animals. These values were obtained with stromuhrs in animals held down on their sides and are considerably lower than those obtained earlier in the anaesthetized lactating goat (30–60 ml./100 g/min) by Linzell (1954). A number of indirect measurements based on the uptake, from analysis of arterial and venous blood samples, of substances appearing in the milk (reviewed by Folley, 1949) have indicated that the ratio of blood flow to milk yield is about 500:1 in the cow and goat. This would indicate absolute flows of at least 30 ml./100 g/min.

In view of these discrepancies it was decided to make new blood-flow measurements at different stages of the reproductive cycle in goats trained to stand quietly without restraint in the laboratory.

METHODS

Two methods, both based on the indicator dilution principle of Stewart, have been found to be applicable to mammary blood-flow measurements in conscious goats subjected to the minimum of restraint (Linzell, 1957). In the first, arterial inflow is measured by injecting Evans Blue dye into the main mammary artery and measuring the concentration–time

curve of the dye in the venous blood. In the second, venous outflow is measured by the thermodilution method of Fegler (1954, 1957), in which cold saline is injected into a main vein and the concentration–time curve of the 'cold' is recorded with a thermocouple downstream. The first method is limited in value because it is difficult to make repeated injections into the mammary artery without disturbing the animal or subjecting it to considerable surgical preparation. The venous outflow method is easily repeatable over long periods, but since there are two main veins draining each of the two glands forming the udder, one (external pudic) as inaccessible as the artery, total venous outflow cannot be measured easily in all animals. However, in many multiparous goats all the venous blood leaves via the readily accessible caudal superficial epigastric vein in front of the udder, in the standing position, and the majority of measurements quoted in this paper were made in this way. The methods of selecting animals on this basis for long-term experiments are described in the preceding paper (Linzell, 1960).

In many of the experiments arterial and mammary venous blood samples were taken immediately after flow measurements for the calculation of mammary uptake of O_2, glucose and volatile fatty acids, and for the calculation of blood flow indirectly from the clearance of Ca.

General procedures

At a preliminary operation under cyclopropane and oxygen anaesthesia a carotid artery and the caudal superficial epigastric vein ('milk vein') on one side were exteriorized as skin-covered loops. At the same time the vessels crossing between the two halves of the udder were divided and their regrowth prevented by careful oversewing with the median suspensory ligament or by separating the two halves permanently with simple skin flaps. In early experiments polythene sheets were sewn between the glands. However, in the course of months, the fibrous tissue around the plastic gradually contracted so that the sheet was tightly compressed into a small package and ceased to separate the udder halves entirely. In none of the animals, however, was there regrowth of large anastomotic vessels across the mid line.

All measurements were made in goats trained to stand in a stand in the laboratory, where they were held only by a neck yoke which allowed them to move their heads in all directions and to feed. The animals were thoroughly accustomed to the laboratory and the experimenters before the experiment began and walked into the stand quite willingly. They stood quietly (often eating or ruminating) during the measurements and were not held in any way. Their heart rates were not significantly different from when they were standing in the field or animal house. No painful procedures were associated with the laboratory because the vein and carotid loops were locally anaesthetized with 2 % lignocaine or 2 % procaine in oil before entering. On a few occasions, when the local anaesthesia was incomplete, puncture of the carotid loop in particular was resented by some animals who reacted by bleating and stamping their feet. Since on all other occasions the animals stood quietly eating whilst the injection, sampling and thermocouple needles were inserted, it can be concluded that the flows were measured in completely undisturbed animals. Some animals did not like being left alone and bleated when the experimenters left the room. The presence of a stranger in addition to the experimenter did not appear to upset them. These precautions were taken because it has been shown in the cow that the disturbance of obtaining arteriovenous samples without local anaesthesia may seriously alter the blood composition, chiefly in respect of haemoglobin and fat (Graham, Kay & McIntosh, 1936; Shaw & Petersen, 1939). The maximum arteriovenous difference for haemoglobin or haematocrit was 3 % and on average was less than 0·5 % in these experiments. Furthermore, no significant difference in composition was found in two venous samples drawn successively over about 2 min, or between samples taken from mid stream or from the periphery of the vein.

As an additional check on the possible effect of the experiments upon the blood flow, the milk yields of the two halves of the udder were recorded separately throughout the entire

lactation period, whereas the blood flows were measured on one side only. Blood flow measurements, even when carried out many times a day, had no immediate effect upon the milk yield of the experimental or the control gland. Furthermore, the total yields of the two halves during the lactation differed by no more than was found in unoperated animals from the same herd.

Arterial blood flow was measured by the injection of 1 ml. 0·5–1·0 % Evans Blue into the main artery of one gland and recording the concentration–time curve of the dye in successive samples of venous blood drawn from the 'milk' vein. The dye concentration was measured either in the plasma or whole blood in a spectrophotometer at 620 mμ. The method was checked in isolated perfused goats' udders by comparing the estimated flow with the venous outflow measured directly and with the arterial inflow calculated from the calibration of the perfusion pump carried out after the experiment. The accuracy was 104 ± 4 % (S.D.). When the glands were perfused *in situ*, in anaesthetized goats supported in the standing position,

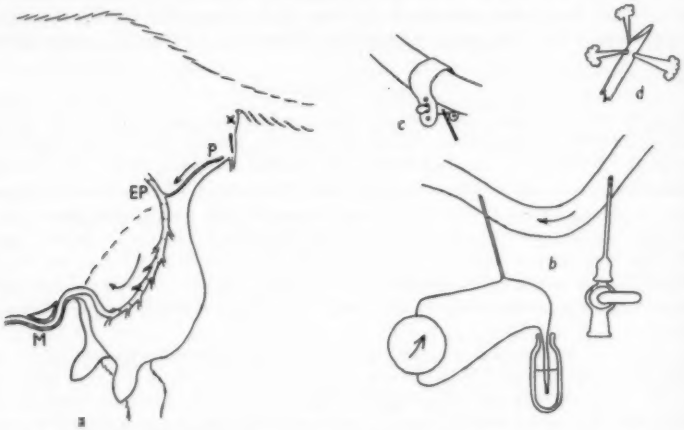


Fig. 1. Method of measuring venous outflow from one mammary gland of the standing goat by the thermo-dilution method, with an exteriorized 'milk' vein (M). (*a*) Disposition of main vessels; during recording the external pudic (EP) and perineal (P) are clamped manually. (*b*) Arrangement of injection needle and needle thermocouple. (*c*) Method of fixing thermocouple to the loop. (*d*) Tip of injection needle giving three jets.

the accuracy was 103 ± 20 %. The artery is difficult to puncture without disturbing the animal, and when exteriorized as a skin-covered loop, the tension necessarily involved and the movement of the udder cause the vessel to close down and atrophy. This had no ill effects on the gland because of the many collaterals which quickly enlarged. In one out of three animals the artery was successfully exteriorized by transplanting one half of the udder, with its main vessels and nerves, to the inside of the thigh so that movement was considerably lessened. In other animals repeated intra-arterial injections were made by cannulating the constant side branch of the main artery that supplies the skin at the side of the udder. In spite of repeatedly washing through with heparin, clotting occurred after a few days.

Venous blood flow was measured by injecting saline (0·2–0·6 ml.) at room temperature into the 'milk' vein loop and recording the change of temperature downstream with a needle thermocouple (Fig. 1*b*). The needle for the injection of saline was blocked at the tip and had

three small holes drilled near the end so that a forceful injection mixed the saline as thoroughly as possible with the blood (Fig. 1*d*). The thermocouple was made of copper constantan 36 s.w.g. wires and the hot junction was welded to the tip of a hypodermic needle, which was held in the mid stream of the vein 5–9 cm from the injection needle by a movable shank and rubber strap (Fig. 1*c*). No solder was used in the construction of the hot or cold junctions of the thermocouple, and the switches, wires and terminals were made of copper. The injection needle, with a Perspex tap of low dead space, and the thermocouple needle and leads were so light and attached in such a way that the animal was quite free to move without disturbing itself or the apparatus. The recording galvanometers were of 1 sec period, slightly less than critically damped and had a sensitivity in use of 33–35 mm/° C. They were calibrated every time they were used against N.P.L. thermometers calibrated to 0·1° C. The records were photographed on moving paper and the area under each response to the passage of cooled blood measured with a planimeter accurate to 0·3 %. The blood temperature and that of the injected saline were recorded just before each injection so that the quantity of injected 'cold' could be determined and the flow calculated from this and the concentration–time curve of 'cold' (Fegler, 1954, 1957).

When the method was used to measure the steady flow of water at 36–40 °C through rubber tubing of the same size as the vein, the accuracy was 99 ± 5 % (S.D.). In a conscious lactating goat the accuracy was 105 ± 11 % as checked by making injections whilst the venous outflow was being collected from a T cannula temporarily inserted into the vein distal to the point of injection. The method was reliable when checked with two needle thermocouples inserted at the same place and when they were a few centimetres apart, but some difficulties were encountered in non-lactating animals, owing to the large size of the superficial epigastric vein. The diameter of the exteriorized vein was 0·8–1·2 cm and during lactation it carried flows of 300–1000 ml./min. The blood temperature was 38·5–39·5° C and did not vary more than 0·1° C in different parts of the stream, even though the flow was lamirar as revealed by the injection of radio-opaque fluids. In non-lactating animals the flow was only 50–150 ml./min, but since the vein is below heart level and sometimes carried non-mammary blood its diameter did not decrease. Under these conditions with the lower flows the blood stream did not always completely fill the vein. Radio-opaque fluids sometimes clung to one wall and the flow profile was often erratic. Relatively stagnant pockets were found where the blood temperature was 0·5–3° C below the hottest point, so that the positioning of the thermocouple then became critical. Below 50 ml./min in such large veins the slow transit time allowed heat exchange to occur and the method was unreliable.

Validation and comparison of methods. Venous outflow from the 'milk' vein was measured directly in two goats (one lactating and the other dry) via a T cannula inserted in the vein under local anaesthesia. The side arm of the cannula had two parallel vertical tubes one of which was used to measure the venous pressure and the other for collecting the blood. Flow was measured by simultaneously clamping the vein beyond the cannula and opening the collecting tube, making sure that during collection the venous pressure remained at its previous level. In both goats other tests showed that the total venous outflow from one gland was flowing through this vein in the standing position. The recorded flows (45 ml./100 g/min for the lactating animal and 28 for the dry one) agree well with the mean values in Table 1. As an additional check the venous outflow (thermodilution) was compared with the arterial inflow (dye dilution) in two lactating goats. In the first, which was selected because total venous outflow was not via the 'milk' vein, the arterial inflow was 31 % greater than the 'milk' vein flow. In the other goat, in which it was believed that the total venous outflow was being measured, the arterial inflow (303 ± 37 ml./min) over a period of 2 hr agreed well with the flow measured in the 'milk' vein (329 ± 52 ml./min).

Gland size. Estimates were made of the weights of the udders by displacement of water after removing the milk as thoroughly as possible with oxytocin, or in the case of the smaller udders of dry animals by taking an impression in calcium alginate (Zelex, Amalgamated

Dental Co.), making a plaster of Paris cast from the impression and getting the volume from the cast. The specific gravity of udder tissue was found to be 1·032–1·045 for lactating animals and 1·03 and 1·054 in two dry pregnant ones. When checked on seven lactating animals that were killed immediately after the measurements, the maximum error encountered was 12%, the mean error 3% and in four cases the estimate was within 1% of the actual weight. In the case of dry non-pregnant animals three had udder sp.gr. of 1·05, 1·07 and 1·16, but in a fourth it was 1·0 because in this, a fat animal, the udder was over 50% adipose tissue. Although a great amount of fat can be detected by palpation and the animals used in this work were not excessively fat, the estimates of the weight of mammary tissue must be considered less accurate in dry animals. In these the maximum error encountered was 25% and the mean error 8%.

Analytical methods

Blood. This was analysed at once for O_2 and CO_2 in the Van Slyke manometric apparatus; for haemoglobin as cyanmethaemoglobin in a spectrophotometer at 642 mμ; for plasma sugar by Somogyi's (1952) method with Nelson's (1944) precipitant; and for calcium and magnesium, by titration of the oxalate and ammonium phosphate salts with EDTA (Wilson, 1955). Analyses were also sometimes made for volatile fatty acids (VFA) (Annison, 1954), inorganic phosphorus (Fiske & Subbarow, 1925 and later Berenblum & Chain, 1938), and packed cell volume by centrifuging at 15000 *g* for 15 min.

Milk. A portion of the total daily milk yield from the experimental side on the day of flow measurement was analysed at once for lactose (Somogyi's reagent after Grimbleby's (1956) precipitation) and for total solids, ash, fat (Rose–Gottlieb) and calcium from the ash after storage at −20° C.

RESULTS

The rate of blood flow through a single mammary gland was found to vary in fourteen conscious goats from 50 to 1000 ml./min, depending upon the stage of the reproductive cycle. When related to the estimated weight of tissue the range was 15–66 ml./100 g/min (Table 1). The flows during lactation were considerably higher than those previously recorded by Jung (1932*a*, *b*; 1933) and Vladimirova (1955), both of whom held the goat down on its side and inserted stromuhrs into the main external pudic artery to the mammary gland under local anaesthesia (the ethyl chloride used by Jung may not have been fully effective). Although it will become apparent that, considered with average arteriovenous differences of milk precursors, the higher flows recorded in this work are the minimum values that could account for the milk formed, some preliminary experiments were done under the conditions used by Jung and by Vladimirova. It was found that holding a goat on its side lowered the mammary blood flow by at least 50% in both lactating and dry animals, as compared with the flows measured at the same time on the previous day (Fig. 2). It will be seen that under effective local and epidural anaesthesia the lowered blood flow was related more to the position of the animal than to the surgical procedure. However, under all forms of anaesthesia dissection of the mammary artery caused it to go into spasm. The fall in flow due to the animal being held down seems likely to be due to stimulation of the sympathetic nervous

TABLE 1. Summary of observations made on 13 goats at different stages of the reproductive cycle; range, (no. of observations), mean ± S.E. of mean. Each observation was made during one session in the laboratory and is the mean of several blood-flow measurements but only one pair of blood samples

	Lactating		Non-lactating	
	More than 50 ml./100 g/day	Less than 50 ml./100 g/day	Pregnant	Non-pregnant
A–V diff.				
O_2 ml./100 ml.	2·4–7·5 (139)	2·8–6·6 (13)	1·3–7·2 (38)	2·0–5·0 (4)
	4·88 ± 0·085	4·6 ± 0·42	3·9 ± 0·18	3·7
Glucose	5–53 (148)	1–22 (21)	+9–20 (43)	+9–5 (4)
(mg/100 ml.)	19·4 ± 0·78	8·6 ± 1·3	5 ± 0·71	1
VFA (mM)	0·42–1·65 (20)	0·4–1·3 (5)	0·25–0·88 (11)	0·33 (1)
	0·95 ± 0·08	0·98 ± 0·165	0·7 ± 0·057	—
R.Q.	0·85–2·4 (130)	0·96–1·5 (20)	0·49–2·1 (31)	0·78–1·25 (4)
	1·24 ± 0·02	1·24 ± 0·011	1·16 ± 0·06	1·01
Blood flow	32–66 (97)	20–44 (10)	15–43 (30)	19–39 (6)
(ml./100 g/min)	47 ± 0·92	31·5 ± 2·2	26 ± 1·5	28 ± 2·8
Uptake				
O_2	1·10–4·47 (76)	0·75–1·86 (6)	0·58–2·02 (28)	0·71, 1·27, 1·96 (3)
(mg/100 g/min)	2·34 ± 0·10	1·42 ± 0·16	1·09 ± 0·057	1·31
Glucose	2·3–26·3 (81)	0·2–3·6 (5)	+1·8–4·1 (28)	0·93, 1·8, +1·7 (3)
(mg/100 g/min)	9·0 ± 0·5	2·2 ± 0·76	0·85 ± 0·25	0·34
Acetate	0·9–3·7 (12)	0·6–2·2 (5)	0·5–1·4 (9)	—
(mg/100 g/min)	2·3 ± 0·23	1·6 ± 0·31	0·8 ± 0·1	—

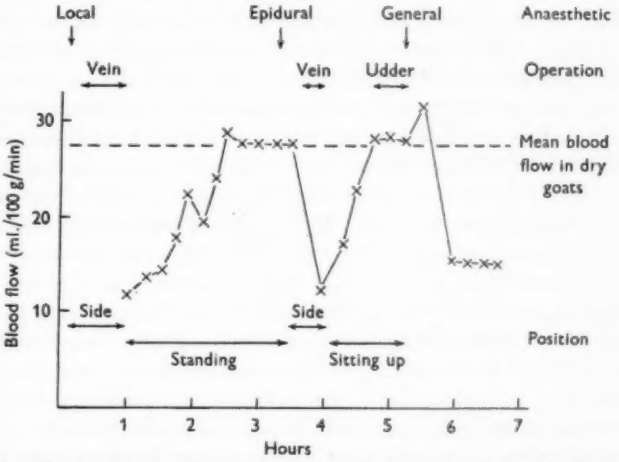


Fig. 2. Effect of posture, anaesthesia and operation on mammary blood flow in a non-lactating goat. The flow from one gland was measured directly at the existing venous pressure via a T cannula inserted in the caudal superficial epigastic vein. The animal received chlorpromazine 2 mg/kg 1 hr before the experiment and heparin 1000 i.u./kg at the start. The flow fell markedly only when the animal was held on its side. The minor operation (cannulation of 'milk' vein) in this position was not responsible because a more extensive operation (separation of two halves of udder) in comfortable sitting position had no effect. Notice the slow recovery after the fall in flow.

system. It is well known that ruminant animals are extremely uncomfortable on their sides, chiefly because they cannot belch so that the rumen quickly becomes distended with the gas constantly produced by fermentation. It has been shown previously that mammary blood vessels are very sensitive to adrenaline and noradrenaline (Hebb & Linzell, 1951) and that the sympathetic adrenergic vasomotor nerves can stop blood flow entirely during stimulation (Linzell, 1950, 1953). In this work it has been found in trained animals standing quietly that intravenous adrenaline 0·8–1·2 μg/kg/min reduces mammary blood flow almost to zero.

A further finding shown in Fig. 2 was that after being held down, although the animals stood quietly eating as soon as they were released, the blood flow did not recover for 1–1·5 hr. After surgical operations in this position under general anaesthesia (e.g. making a carotid loop), the mammary blood flow remained 40–62 % of normal for as long as 4 hr after recovery from the anaesthetic.

Variations in blood flow

Minute-to-minute. The method of venous outflow measurement used integrates the flow over a period of 5–10 sec and has an error *in vitro* of 5 % (S.D.) of the mean. *In vivo* this was apparently increased to 10 %, but it is possible that part of this additional fluctuation may be genuine, because the method could only be checked accurately by collecting the venous outflow over a period of 30 sec. In the majority of experiments where flow was measured 2–20 times over periods of 3–60 min the variation was of the order of 10 % (S.D.) In a small number of observations the flow varied 1 % or less or showed larger variations up to a maximum of 20 %.

Hour-to hour. No clear evidence of significant variation was obtained. Observations were made in one goat at different hours of the day and night on 6 days between the 23rd and 34th weeks of lactation, when the milk yield was almost stationary. The mean flows for each day were identical but the standard deviation of observations varied from 8 to 38 % of the mean. The highest flows (120 % of the mean) were encountered just before and after the morning and afternoon milkings, at which time the goat was also fed, and the lowest (75 % of the mean) midway between milkings. Therefore the majority of observations reported in this paper were made just after the morning milking (9.30 a.m.) and again about 3·5–4 hr later. However, over this animal's next lactation the morning flow was only greatest on 8 out of 14 days and when the results from all the goats were analysed at the end of the experiment no significant difference was found.

Day-to-day. The blood flow measured on one day (mean of morning and midday estimates) averaged 99 $\pm$ 10 % of that measured 2–4 days earlier in the same way.

Month-to-month. Since the flows measured twice in one day and on 2 days in one week did not differ significantly, the values were pooled and used as an estimate of the blood flow during that week. Analysis of arterial and mammary venous blood samples obtained at each session gave four estimates of R.Q. and O_2, glucose and sometimes volatile fatty acid (VFA) uptake. The average daily milk yield for the week and the empty weight of the gland were also recorded. In five animals this was repeated monthly during the normal lactation (some results had to be discarded because total blood flow was not always measured), a sixth animal was followed for 2 years over 2 pregnancies and 1 lactation, and a number of other estimates were made in the remaining eight goats at all stages of the reproductive cycle. The results are summarized in Table 1.

It will be seen, as might be expected, that the blood flow, the arteriovenous differences, the R.Q. and the calculated uptakes of O_2, glucose and VFA were all greater in lactating than in dry animals. However, in non-lactating animals there was little significant difference between non-pregnant and pregnant animals, except possibly that the uptake of glucose and the R.Q. were higher in pregnant animals. Observations made during lactation have been divided into those from fully lactating animals (more than 50 ml. milk/100 g tissue/day) and those from animals in declining lactation (less than 50 ml.). This figure was chosen because below this level lactation ceased quickly in most animals. Table 1 shows that the blood flow, O_2 consumption and the uptake of glucose and VFA were all lower at this low milk yield. However, to some extent the grouping of the observations in this way is misleading, because it obscures variations occurring at different stages of pregnancy and lactation (Figs. 3 and 4). During lactation in each animal the blood flow, O_2, glucose and VFA consumption all varied with the milk yield and a similar dependence was seen between the average daily milk yield and the blood flow and glucose uptake per 100 g tissue in all the goats (Fig. 5).

The ratio of blood flow to milk yield

Mammary blood flow has been estimated in the past from the plasma clearance of milk precursors on the Fick principle and the results expressed as the ratio of blood flow to milk yield. Estimates have varied from 200 to 1000:1 but a figure of about 500:1 is generally accepted (see Folley, 1949). In this work it has been possible to calculate the ratio directly from observed blood flows and indirectly from arteriovenous differences.

Direct estimates. These show that the ratio of blood flow to milk yield is not fixed but varies inversely with the rate of milk secretion and is thus a measure of efficiency (Fig. 6). In Fig. 6 the curve has been calculated from the regression of Fig. 5*a* and it will be seen that actual observations

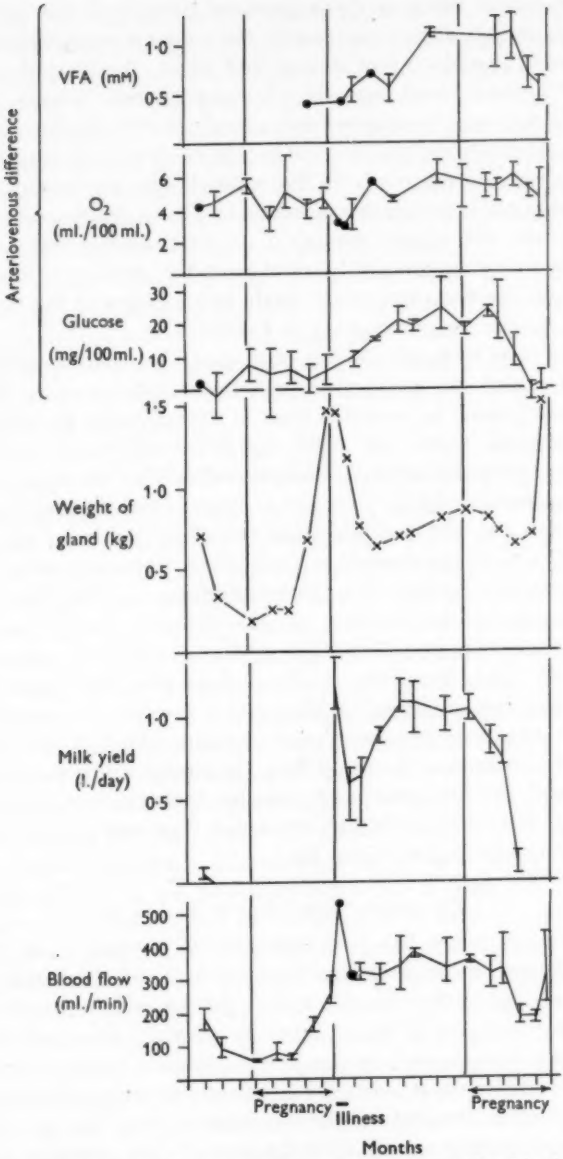


Fig. 3. Variations during the reproductive cycle of one goat (Bessie); right gland only; mean and range of observed data during a week. The fall in milk yield due to a mild illness at the start of lactation is abnormal (see Fig. 4).

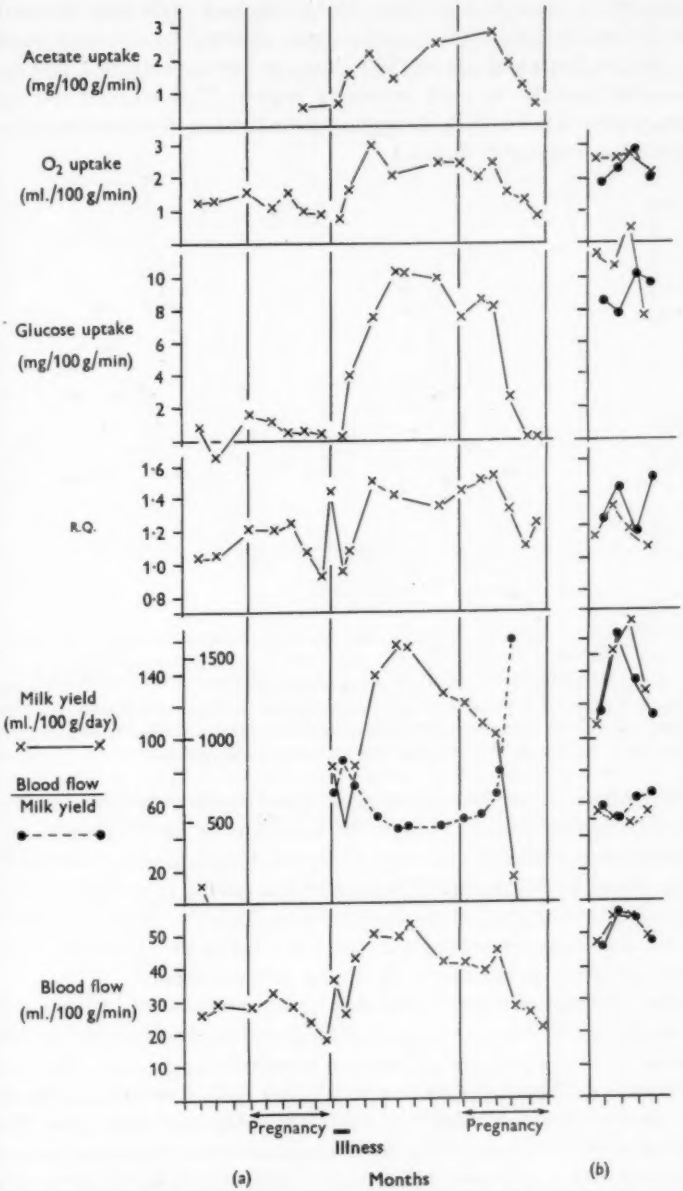


Fig. 4(*a*). Calculated data derived from Fig. 3. The values in early lactation are abnormal owing to mild illness and typical values for two other goats (Joan and Gertrude) are shown in (*b*). Volatile fatty acid uptake is calculated as acetate.

fall reasonably close to this line. At the highest yield recorded in these goats (172 ml./100 g/day) the average ratio of blood flow to milk yield was 460:1 but at low yield (50 ml./100 g/day) it was over 1000:1 and rapidly approached infinity as milk secretion ceased. This marked decrease in efficiency associated with declining lactation further justifies the separation of these observations in Table 1.

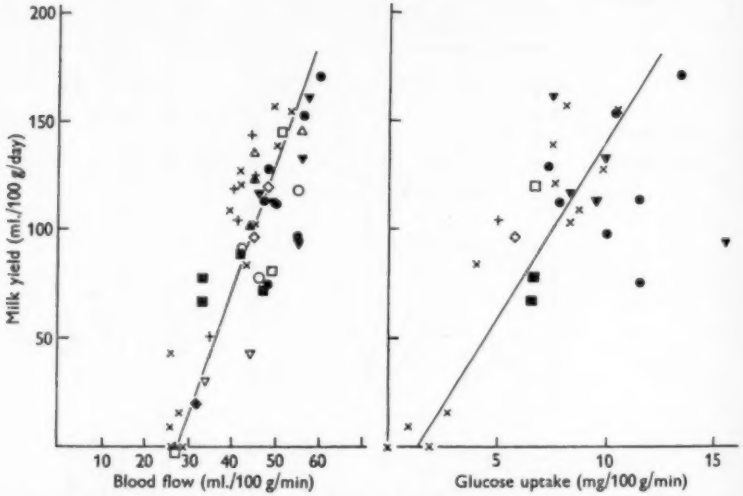


Fig. 5. Variations in blood flow and glucose uptake with the rate of milk secretion. Each point is the mean of 4 estimates in one week; twelve goats. Goat Bessie ×, Joan ●, Gertrude ▲. The lines are calculated from regressions.

It is of interest that the ratio of blood flow to milk yield also varied in the expected way at different stages of lactation in each animal and also changed during a fall in yield owing to illness. The yield of the animal shown in Figs. 3 and 4*a* fell shortly after parturition (owing to a uterine infection) at a time when the yield normally increases (Fig. 4*b*). However, the blood flow fell less than the milk yield, the ratio being 880:1, indicating a decreased efficiency in the secretory ability of the tissue.

Indirect estimates. Shaw & Petersen (1940) recommended the use of the arteriovenous difference of Ca, since all milk Ca must come from the plasma, whereas, for example, not all lactose comes from glucose. The average arteriovenous difference of Ca was $0{\cdot}22 \pm 0{\cdot}01$ (S.E. of mean) mg/100 ml. for goats giving more than 50 ml. of milk/100 g /day, and $0{\cdot}15 \pm 0{\cdot}04$ for non-lactating animals. These values did not appear to increase with the interval between milkings as Shaw & Petersen (1940) found in the cow, but unfortunately were small in relation to the error of the method ($0{\cdot}28 \pm 0{\cdot}01$

mg/100 ml.). Therefore, as might be expected, the ratio of blood flow to milk yield calculated from individual figures varied widely and even the mean of 2–4 observations in a day varied from 30 to 150 % of direct estimates

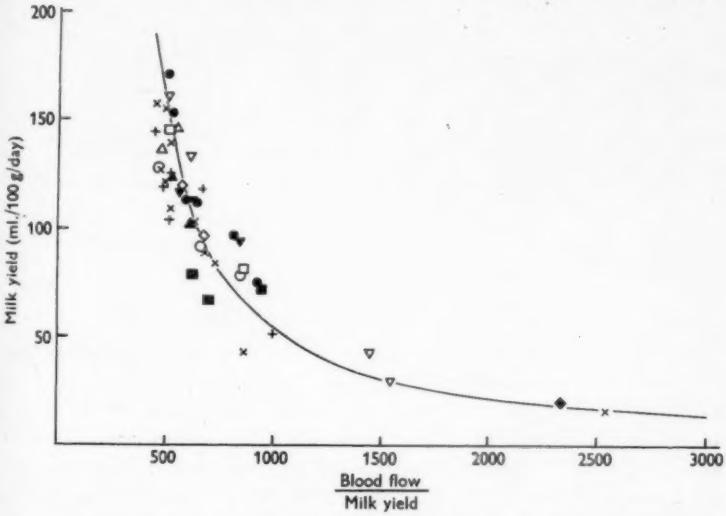


Fig. 6. Relation between blood flow/milk yield and level of milk secretion, showing that this is a measure of efficiency. The curve is calculated from the regression line in Fig. 5 and the points are weekly averages (twelve goats).

TABLE 2. A quantitative comparison of the major milk constituents with their probable plasma precursors in the goat. Based on the following average figures: milk yield 100 ml./100 g/day; blood flow 45 ml./100 g/min; lymph flow 50 ml./100 g/day; milk composition (%), fat 4·76, lactose 4·7, total N 0·53. The arteriovenous differences for fat and amino N have been taken from Lintzel (1934). The calorific value of milk was calculated by the method of Perrin (1958)

Substance	Mammary uptake (g/100 g tissue/day)		Milk output (g/100 g tissue/day)
Calcium	0·142		0·143
Glucose	10·3	lactose	4·7
Fat	3·2		4·76
Acetate	3·2		—
Amino N (see p. 509)	0·65	total N	0·52
Calories (kcal)	103	81	
O_2	3·37 l. ≡ 17 kcal		—

made at the same time. Nevertheless, the ratio of blood flow to milk yield (645:1) calculated from the mean figures in Table 2 agrees well with that observed directly (660:1). Similarly, in declining lactation the calculated ratio from Ca uptake (1700:1) is of the same order as that observed directly (2000:1).

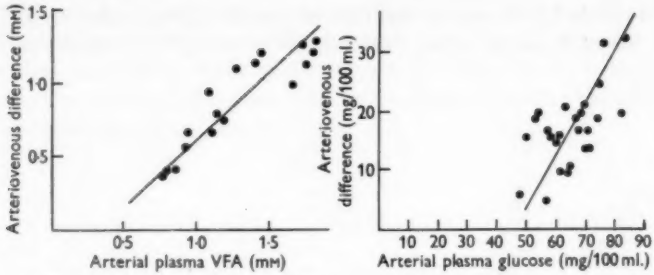


Fig. 7. Variations of arteriovenous differences of volatile fatty acids and glucose with arterial plasma level of goat Bessie during lactation. The lines are calculated from regressions.

DISCUSSION

The blood flows recorded under these conditions are several times higher than those previously measured directly by Jung (1932*a*, *b*, 1933) and Vladimirova (1955). It is believed that the higher values obtained in this work more nearly approach the normal, for the following reasons. The measurements were made in undisturbed animals and it has been shown that the methods used previously reduce the flow. Moreover, the methods used here for long periods did not significantly affect the milk yield of the gland used for measurement as compared with the opposite gland of the same animal, or as compared with other animals of the same herd. When considered with arteriovenous differences measured at the same time (which agree well with previous figures), the calculated uptake of Ca is just enough to account for that appearing in the milk. The O_2 consumption per gram of tissue is similar to that encountered in slices of goat mammary gland by Folley & French (1949) and glucose consumption is similar to that of slices of guinea-pig mammary gland (Venkataraman & Reithel, 1957).

It must be pointed out that, since the mammary blood vessels also supply the surrounding skin and connective tissue, the figures given here include the blood flow through the teat, the skin of the udder and the suspensory ligaments and connective tissue capsule. In lactating goats mammary tissue forms 84 ± 4 (S.D.) % of the weight of the udder, so that the recorded blood flow must be mainly that through secretory tissue. However, in non-pregnant dry goats mammary tissue may form only 40–60 % of the total weight of the udder when involution is maximal. The figures for total flow through dry glands therefore should be interpreted with this in mind. In pregnant goats the proportion of mammary tissue increases with the growth of the gland.

Previous indirect estimates of blood flow in lactating cows and goats,

expressed as the number of volumes of blood that flow through the mammary gland to form one volume of milk, more nearly agree with the present values than the older direct estimates already referred to. In this work it has been found that this ratio varies with the amount of milk secreted and thus may be a measure of over-all efficiency. It is well known that the udder decreases in size during lactation. In this herd in non-pregnant animals the glands shrink to 25–40 % of their maximum weight in about 10 months, and this is accompanied by a fall in yield per gram of tissue (Fig. 4).

It has been pointed out already that the proportion of mammary tissue is also lower in dry animals. If the skin, teat and connective tissue have a much lower blood flow than the mammary tissue the question arises as to whether an increasing proportion of non-mammary tissue accounts for the apparent fall in efficiency and milk yield per gram of total tissue as the milk yield declines. It seems unlikely that this could cause the whole effect. It has been calculated that the proportion of non-mammary tissue (skin, teat and capsule) of the glands concerned (500–1500 g) might be expected to increase from about 10 to 20 % during the course of the lactation. Figures used in this calculation were an average skin thickness of 0·7–1·0 mm and a teat weight of 35–50 g (this changes little during a lactation). The area of skin and capsule was measured from plaster casts of the glands, where available, or calculated from the volume (assuming the gland to be a hemisphere, a cylinder or appropriate geometrical shape). Actual measurements from other goats killed at known stages of lactation were essentially in agreement. The milk yield per gram of total tissue per day decreases from about 1·7 to zero, whilst the proportion of mammary tissue only falls from 90 to 80 % of the total weight. Clearly there is a real decrease in the amount of milk secreted by each gram of tissue as lactation proceeds. Furthermore, even if it is assumed that the blood flow through the non-mammary tissue is zero then there is still a relation between the yield per gram and the blood flow per gram, and of course in this case the ratio of blood flow to milk yield increases even more markedly as the yield decreases. In fact, it may well be that the blood flow through the skin and teat is relatively high. Both structures have a large number of arterio-venous anastomoses (starch grains 22 μ pass easily through goats' udders only when the skin and teat are present) and the udder skin temperature in lactating goats is 36–38° C. If this is true then the error due to the inclusion of non-mammary tissue in blood flow measurement is much smaller than is already allowed for.

It must be admitted that the changes in mammary gland weight in the goat are partly due to a varying proportion of purely secretory tissue over and above a relatively constant weight of large ducts and cisterns. Although

this must partly account for the variations in milk yield and blood flow, the fact that the milk yield can vary greatly whilst mammary weight changes little shows that the rate of secretion by the alveolar tissue can also change (Fig. 3). This occurs at the very end of lactation in all goats.

It would seem reasonable to conclude, therefore, that the decline in milk yield that occurs during a lactation is due both to a loss of secretory tissue and to a fall in the rate of secretion per cell. Furthermore, even when allowance has been made for the increasing proportion of non-mammary tissue, there is a parallel decrease in efficiency of milk formation in that less milk is formed from each volume of blood passing through the gland.

One may also investigate mammary efficiency by comparing the uptake of milk precursors from the plasma with the amount of the product appearing in the milk in the same time (Table 2). The hazards of equating small arteriovenous differences calculated from samples collected in less than a minute with milk formed during a period of hours must be stressed. This has been already pointed out in the case of Ca, where the arteriovenous difference is very small and, as might be expected, the errors are less in the case of glucose and lactose, where the difference is larger. When calculated from weekly figures of all goats the glucose accounted for as lactate is $55 \pm 5\%$ which agrees well with the value in Table 2 calculated from means.

It will be seen that for Ca the efficiency approaches 100%. However, in the case of daily balances for individual goats in early lactation the Ca uptake was more often insufficient to account for the milk Ca, whereas late in lactation it was more often in excess of that in the milk. This supports the suggestion of Swanson, Monroe, Zilversmit, Visek & Comar (1956) from studies of ^{45}Ca excretion in the milk of cows, that the udder has a variable store of calcium.

It is less easy to do quantitative balances for the major milk constituents because much modern work with isotopes shows that several plasma precursors help to form each substance (Folley, 1956). Nevertheless, milk proteins and lactose are derived mainly from plasma amino acids and glucose, respectively. In the ruminant acetate is the source of the 10% of short-chain fatty acids (C_4–C_{12}) of milk fat and most probably forms acids up to C_{16} as well (i.e. 50% of fat). The remainder is largely derived from plasma lipids.

The remaining figures in Table 2 show that the mammary gland transfers about 80% of the total calories and amino N removed from the plasma into the milk. Only 50% of the glucose can be accounted for as lactose but milk fat could account for 80% of the neutral fat and acetate taken up. It

would seem likely that some acetate and glucose are oxidized, but it is not possible to decide the proportion of each because the R.Q. of lactating tissue is over 1 and the R.Q. for acetate and glucose are identical. The acetate consumption of non-lactating tissue is similar to that of glucose. This suggests that acetate is being oxidized as in most other ruminant tissues, and sufficient is taken up by the lactating tissue to continue this and transform the remainder into fatty acids. Nevertheless, one of the most striking findings in this work is the very great increase in the uptake of glucose during lactation. Whereas O_2 consumption doubles, and acetate consumption trebles, glucose uptake goes up nine times. This is more than sufficient to account for all the lactose and the entire O_2 consumption.

Gaps in our knowledge of glucose metabolism in ruminants have been pointed out by Lindsay (1959). The estimated daily turnover of glucose in a sheep is only 100 g and yet a lactating ewe gives about 80 g of lactose daily in the milk. 200 g of lactose daily would not be unusual for a lactating goat and the present figures indicate that at maximum efficiency at least 270 g of glucose would be taken up by the udder to produce this. These figures emphasize the very great demand for glucose that the onset of lactation suddenly throws on the organism. Although the arterial plasma glucose was slightly higher in lactating (60 mg %) than in dry animals (53 mg %) the figures were not statistically significant. However, as Graham, Jones & Kay (1936) found in the cow, the arteriovenous difference of glucose increased with the arterial level (Fig. 7). Similarly the level of arterial plasma VFA was higher in lactating animals (1·3 mM) than in dry ones (0·89 mM) and, as McClymont (1951) found in the cow, the arteriovenous difference was also related to this level. These figures emphasize the avid uptake of glucose and acetate by the mammary glands.

It is not surprising that the present figures show that the metabolic rate of the lactating mammary gland is similar to that of the brain and approaches that of the liver. This is to be expected in an organ that is actively synthesizing. The fact that the blood flow and the uptake of milk precursors do not fluctuate widely during the 24 hr supports the recent view that ordinarily milk secretion is a steady continuous process (see Linzell, 1959). Even in dry animals the total udder blood flow is equivalent to that through skin during maximal vasodilatation, suggesting that mammary tissue is always metabolically relatively active.

SUMMARY

1. Mammary-gland blood flow, weight, milk yield and arteriovenous differences of O_2, Ca, glucose and volatile fatty acids have been measured at all stages of the reproductive cycle in goats trained to stand in the laboratory.

2. The mean blood flow was 28 ml./100 g/min in dry goats and 45 ml./100 g/min in lactating animals giving 100 ml. milk/100 g/day. Holding goats on their sides, as was done by previous workers who recorded much lower figures, significantly reduced the mammary blood flow.

3. Blood flow variation from minute to minute was 10% of the mean (S.D.), and was not significantly greater from hour to hour or from day to day. During lactation the blood flow varied with the milk yield.

4. The mean arteriovenous differences and instantaneous R.Q. agree well with those of previous workers. The mean ratio of blood flow to milk yield (650:1) calculated from the Ca difference and the milk Ca agrees with earlier estimates and with directly observed values, but was found to increase with a fall in milk yield and is thus a measure of efficiency.

5. During lactation O_2 consumption doubles, the uptake of volatile fatty acids trebles but glucose uptake increases nine times.

6. On the average 100% of the Ca, and 80% of the N and calories removed from the blood appear in the milk. Similarly 80% of the neutral fat and volatile fatty acids taken up are accounted for in milk fat but only 50% of the glucose appears as lactose.

7. The importance of glucose in the metabolism of lactating ruminants is stressed.

I am grateful to Mr I. R. Fleet for efficient and enthusiastic technical assistance, to Mr G. Bull for help with blood and milk analyses and to Dr D. C. Hardwick for many helpful discussions.

REFERENCES

ANNISON, E. F. (1954). Studies on the volatile fatty acids of sheep blood with special reference to formic acid. *Biochem. J.* **58**, 670–680.

BERENBLUM, I. & CHAIN, E. (1938). An improved method for the colorimetric determination of phosphate. *Biochem. J.* **32**, 295–298.

CARGILL-THOMPSON, H. E. C., DRURY, A. N., HARDWICK, D. C., LINZELL, J. L. & TUCKER, E. M. (1958). The blood flow and milk yield of goats' udders perfused with artificial media. *J. Physiol.* **143**, 74–75*P*.

FEGLER, G. (1954). Measurement of cardiac output in anaesthetized animals by a thermodilution method. *Quart. J. exp. Physiol.* **39**, 153–164.

FEGLER, G. (1957). The reliability of the thermodilution method for the determination of the cardiac output and the blood flow in central veins. *Quart. J. exp. Physiol.* **42**, 254–266.

FISKE, C. H. & SUBBAROW, Y. (1925). The colorimetric determination of phosphorus. *J. biol. Chem.* **66**, 375–400.

FOLLEY, S. J. (1949). Biochemical aspects of mammary gland function. *Biol. Rev.* **24**. 316–354.

FOLLEY, S. J. (1956). *The Physiology and Biochemistry of Lactation*. London and Edinburgh: Oliver and Boyd.

FOLLEY, S. J. & FRENCH, T. H. (1949). The intermediary metabolism of the mammary gland. I. Respiration of lactating mammary gland slices in presence of carbohydrates. *Biochem. J.* **45**, 117–125.

GRAHAM, W. R. (1937). The utilization of lactic acid by the lactating mammary gland. *J. biol. Chem.* **122**, 1–9.

GRAHAM, W. R., JONES, T. S. G. & KAY, H. D. (1936). Precursors in cows' blood of milk fat and other milk constituents. *Proc. Roy. Soc.* B, **120**, 330–346.

GRAHAM, W. R., KAY, H. D. & MCINTOSH, R. A. (1936). A convenient method for obtaining bovine arterial blood. *Proc. Roy. Soc.* B, **120**, 319–329.

GRIMBLEBY, F. H. (1956). The determination of lactose in milk. *J. Dairy Res.* **23**, 229–237.

HEBB, C. O. & LINZELL, J. L. (1951). Some conditions affecting the blood flow through the perfused mammary gland, with special reference to the action of adrenaline. *Quart. J. exp. Physiol.* **36**, 159–175.

JUNG, L. (1932*a*). Importance de la circulation à travers la mamelle au repos ou en lactation chez la chèvre. *C.R. Soc. Biol., Paris*, **109**, 737–739.

JUNG, L. (1932*b*). Particularités de la circulation mammaire chez la chèvre en lactation. *C.R. Soc. Biol., Paris*, **109**, 1052–1053.

JUNG, L. (1933). Intensité de la circulation mammaire. *Lait*, **13**, 307–315.

LINDSAY, D. B. (1959). The significance of carbohydrate in ruminant metabolism. *Vet. Rev. Annot.* **5**, 103–128.

LINTZEL, W. (1934). Le chimisme de la formation du lait. *Lait*, **14**, 1125–1130.

LINZELL, J. L. (1950). Vasomotor nerve fibres to the mammary glands of the cat and dog. *Quart. J. exp. Physiol.* **35**, 295–319.

LINZELL, J. L. (1953). Internal calorimetry in the measurement of blood flow with heated thermocouples. *J. Physiol.* **121**, 390–402.

LINZELL, J. L. (1954). Some observations on the use of the perfused lactating mammary gland. *Rev. canad. Biol.* **13**, 291–298.

LINZELL, J. L. (1957). The measurement of udder blood flow in the conscious goat. *J. Physiol.* **137**, 75–76*P*.

LINZELL, J. L. (1959). Physiology of the mammary glands. *Physiol. Rev.* **39**, 534–576.

LINZELL, J. L. (1960). Valvular incompetence in the venous drainage of the udder. *J. Physiol.* **153**, 481–491.

MCCLYMONT, G. L. (1951). Volatile fatty acid metabolism of ruminants with particular reference to the lactating bovine mammary gland and the composition of milk fat. *Aust. J. agric. Res.* **2**, 158–180.

NELSON, N. (1944). A photometric adaptation of the Somogyi method for the determination of glucose. *J. biol. Chem.* **153**, 375–380.

PERRIN, D. R. (1958). The calorific value of milk of different species. *J. Dairy Res.* **25**, 215–220.

SHAW, J. C. & PETERSEN, W. E. (1939). Blood volume changes in the mammary gland. *Proc. Soc. exp. Biol., N.Y.*, **42**, 520–524.

SHAW, J. C. & PETERSEN, W. E. (1940). The fat metabolism of the mammary gland. *J. Dairy Sci.* **23**, 1045–1056.

SOMOGYI, M. (1952). Notes on sugar determination. *J. biol. Chem.* **195**, 19–23.

SWANSON, E. W., MONROE, R. A., ZILVERSMIT, D. B., VISEK, W. J. & COMAR, C. L. (1956). A study of the variations in secretion of Ca^{45} by the mammary glands of dairy cows. *J. Dairy Sci.* **39**, 1594–1608.

VENKATARAMAN, R. & REITHEL, F. J. (1957). Studies on lactose synthesis in mammary gland slices. *Arch. Biochem. Biophys.* **70**, 205–209.

VLADIMIROVA, A. D. (1955). Reflex regulation of blood supply to the udder. *J. gen. Biol., Moscow*, **16**, 141–155.

WILSON, A. A. (1955). The determination of magnesium and calcium in serum or plasma with ethylene diamine tetra-acetic acid (EDTA). *J. comp. Path.* **65**, 285–290.

Note added in proof

There is some doubt whether Lintzel (1934. *Z. Zücht. B.* **29**, 219–242) reported total amino-acid N. If he did not, then the mammary uptake may be greater than that indicated, and the over-all N-efficiency less.

J. Physiol. (1960), **153**, *pp.* 510–521
With 1 *plate and* 4 *text-figures*
Printed in Great Britain

THE FLOW AND COMPOSITION OF MAMMARY GLAND LYMPH

By J. L. LINZELL

From the Agricultural Research Council Institute of Animal Physiology, Babraham, Cambridge

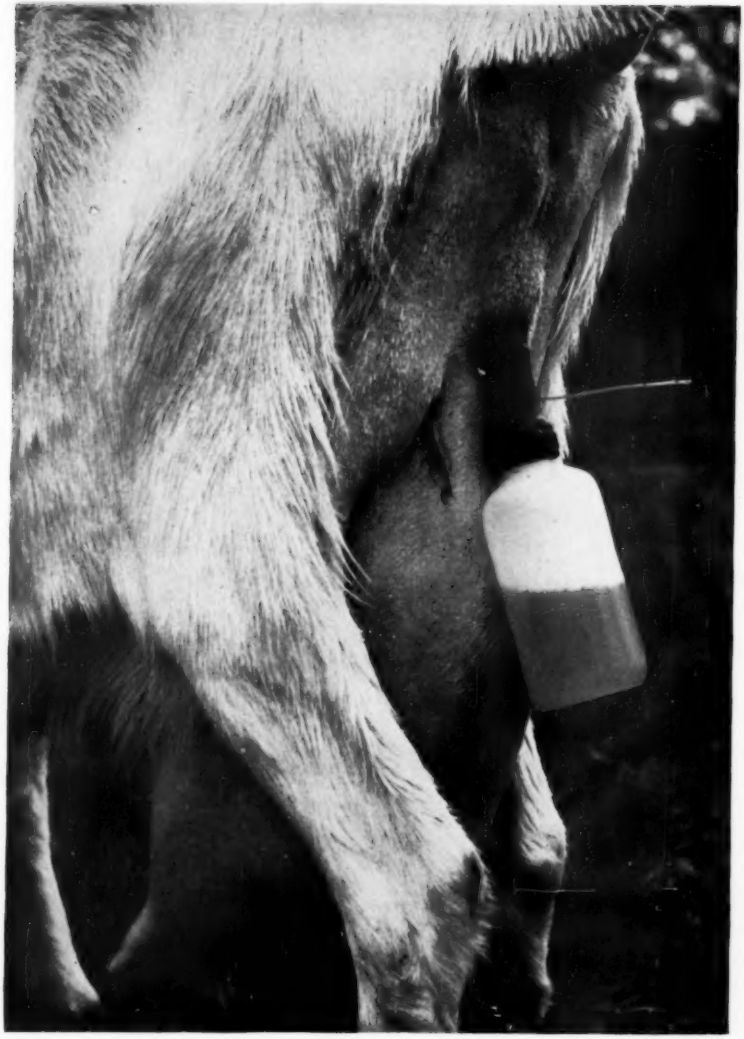
(*Received* 22 *June* 1960)

There are no published figures for the rate of lymph flow from the mammary glands, although a number of authors have stated that it should be considered in the study of the uptake of milk precursors from the blood by the mammary glands. In recent years Belgian workers have suggested the study of mammary lymph composition as a means of investigating mammary gland metabolism and have published some detailed analyses of the lymph taken from the udders of dead cows (Heyndrickx & Peeters, 1958*a*, *b*, 1960; Heyndrickx, 1959). Mammary gland lymph has been collected on a number of occasions in this laboratory over a period of 10 years, chiefly from goats during studies of the transfer of metabolites from blood to milk (Linzell, 1960) and during a study of the perfused goat's udder (Cargill-Thompson, Drury, Hardwick, Linzell & Tucker, 1958; Hardwick, Linzell & Price, 1960). The results are presented now because sufficient data have accumulated to suggest that mammary lymph flow does reflect the metabolic activity of the tissue and also to sound a note of caution, because the composition of the lymph collected from conscious goats differs in some important respects from that obtained from dead cows. In addition, it seems worth recording that large quantities of lymph can be obtained for long periods with minimal disturbance from goats' udders, because this type of preparation may be of use to workers interested in lymph.

METHODS

The lymphatic vessels from the two mammary glands forming the udder in the goat and sheep pass to the superficial inguinal lymphatic glands on each side, from whence a single lymphatic trunk passes into the abdomen through the inguinal canal, parallel and caudal to the external pudic blood vessels. This efferent duct was cannulated with polythene tubing (1–2 mm outside diameter; 20–30 cm long) a few centimetres from the lymph gland (Fig. 1). In some animals there are 2 or 3 efferent lymph trunks; in such cases one was cannulated and the others ligated.

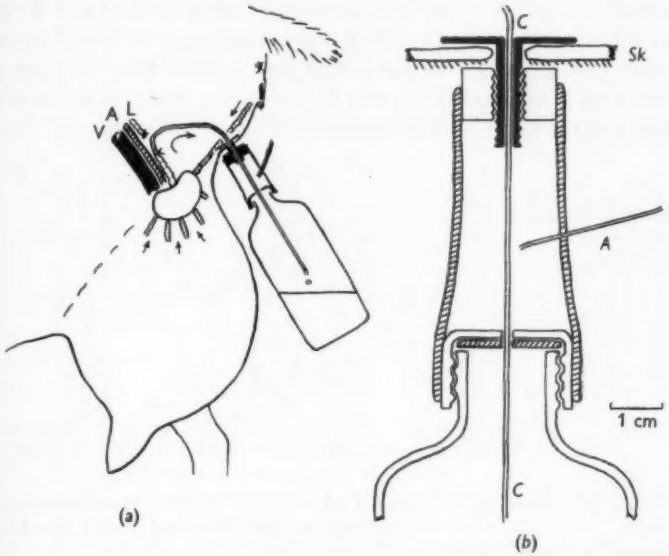
For continuous collection in conscious goats a polythene bag or bottle (100–250 ml.) was fixed to the animal. In early experiments epidural anaesthesia was used (5–8 ml. lignocaine 2% in the lumbosacral space), but this suffered from the disadvantage that some animals staggered about as the anaesthetic wore off and had to be temporarily supported in a sling to prevent the cannula from being dislodged. Later, satisfactory anaesthesia was obtained



J. L. LINZELL

(*Facing p.* 511)

by blocking the udder nerves paravertebrally (Linzell, 1959); in this case there was no paralysis of the hind quarters. A tranquillizing dose of chlorpromazine hydrochloride, 1·70 mg/kg was given ½–1 hr before the operation. The lymph cannula was anchored to the lateral suspensory ligament of the udder when this was stitched up after cannulation, and the distal end was led into the collecting bottle via a stainless-steel cannula, inserted through the skin 5–7 cm from the vulva, with a flexible rubber tube joining it to the cap of the bottle (Text-fig. 1 and Pl. 1). The animal thus had complete freedom and the collecting bottle could be changed without disturbing it or the cannula. The narrow space between the lymph cannula and the skin cannula quickly filled with wound fluid, which clotted and effectively sealed the space so that uncontaminated lymph was obtained. Some of the goats



Text-fig. 1. Diagram of method used for the continuous collection of mammary lymph in conscious goats. (*a*) Position of lymph duct (L) and cannula, external pudic artery (A) and vein (V). (*b*) Scale diagram of flexible rubber connector used to join the polythene collecting bottle to the animal. *C*, Lymph cannula; *Sk*, skin; *A*, air vent. Plastic, white; stainless steel, black; rubber, cross-hatched.

used for continuous collection were also used for mammary blood-flow measurements and had a mammary vein and a carotid artery exteriorized, so that blood samples could also be obtained without disturbance during lymph collection.

Lymph and plasma were analysed by the following methods: Protein, as total N, by a micro-Kjeldahl method; Na and K by flame photometry (EEL Photometer); Ca and Mg, Wilson (1955); Cl, Van Slyke & Hiller (1947); HCO_3, Van Slyke & Sendroy (1928); volatile fatty acids (VFA), Annison (1954); glucose, Nelson (1944) and Somogyi (1952); lactate, Barker & Britton (1957); inorganic P, Berenblum & Chain (1938); amino acids, Hamilton & Van Slyke (1943).

EXPLANATION OF PLATE

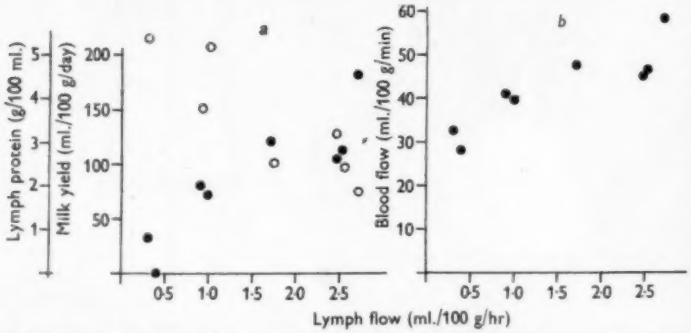
Photograph of the position of the lymph-collecting bottle in a conscious goat.

RESULTS

Mammary lymph flow in conscious goats

Collections were made in 8 Saanen goats for periods varying from 8 hr to 17 days. The volume of lymph collected in non-pregnant animals ranged from 150 to 840 ml./day and the most comprehensive observations are summarized in Table 1.

It must be pointed out that the lymph duct cannulated drains the whole inguinal region including the mammary gland, skin, the teat and the vulva, so that it has to be decided to what extent the lymph obtained represents mammary-gland lymph. It was not at first realized that an appreciable proportion of the lymph from the vulva drains through the superficial inguinal lymph gland at the base of the udder (Text-fig. 1). This was



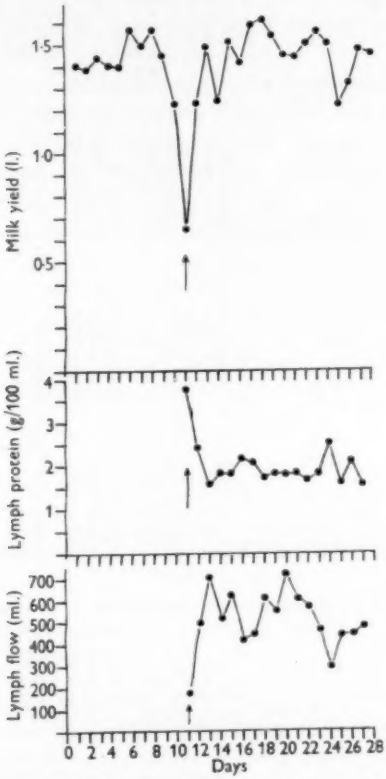
Text-fig. 2. (*a*) Relation of lymph flow (●) to milk yield/100 g tissue and to lymph protein (○). (*b*) Relation of lymph flow to blood flow/100 g tissue. Blood-flow figures from Linzell (1960).

suspected when it was observed that, whereas the lymph flow in dry non-pregnant goats was less than in non-pregnant lactating animals (Text-fig. 2), in pregnancy the flow per gram of tissue remained equal to the highest flows, irrespective of the milk yield, and was often 10–30 % higher. The highest flows recorded (800–1000 ml./day, or 3 ml./100 g/hr) were obtained in one animal in the 3 days before parturition, when the whole perineum was oedematous. In this animal red blood cells appeared in the lymph during parturition and the lymph unexpectedly clotted in the cannula 7 hr later. Next day, following the injection of Evans Blue dye (4 %) around the vulva, 3–4 small lymphatics containing the dye were seen to be running from the vulva towards the superficial inguinal lymph gland in company with the perineal blood vessels. Similar vessels were demonstrated by the same method in other goats, although they were smaller in non-pregnant animals (Text-fig. 1).

TABLE 1. Summary of observations (mean ± S.E. of mean) made during collection of lymph from one mammary gland in each of 4 conscious lactating Saanen goats, together with the analysis of plasma from lactating goats in the same herd. Lymph analyses refer to 24-hr samples. The numbers of animals used for plasma analysis are shown in brackets; mean of 1–40 estimates for each animal. A—arterial; V—mammary venous

Animal		Jill	Pam	Bessie	Gracie	Plasma
Milk yield (ml./day)		1,207 ± 23	626 ± 13	901 ± 34	1,445 ± 31	—
Weight of mammary gland (g)		1,000	600	800	800	—
Length of lymph collection (days)		7	15	7	17	—
Lymph flow (ml./day)		415 ± 14	357 ± 12	488 ± 14	520 ± 16	—
Lymph flow (ml./100 g/hr)		1·72	2·47	2·54	2·71	—
Na	(m-equiv/l.)	150·3 ± 1·1	150 ± 0·9	151 ± 1·8	148 ± 1·2	153 ± 1·3 (13)
K		4·8 ± 0·16	4·47 ± 0·05	4·66 ± 0·15	4·46 ± 0·08	4·23 ± 0·08 (13)
Ca		2·61 ± 0·2	3·09 ± 0·15	2·71 ± 0·2	2·68 ± 0·21	4·38 ± 0·21 (10)
Mg		2·33 ± 0·21	2·06 ± 0·07	1·88 ± 0·07	2·02 ± 0·09	2·29 ± 0·33 (10)
Cl		118·6 ± 1·1	114·5 ± 0·95	120·4 ± 0·8	112·9 ± 0·71	109·25 ± 0·8 (12)
HCO_3		23·6 ± 0·7	27·6 ± 0·37	25·0 ± 0·9	26·0 ± 0·65	A 24·1 ± 0·5 } (10) V 26·1 ± 0·6 }
Inorganic P		2·13 ± 0·17	3·10 ± 0·07	3·25 ± 0·17	1·9 ± 0·04	3·50 ± 0·22 (7)
Lactate		0·68 ± 0·12	0·78 ± 0·10	0·44 ± 0·11	0·35 ± 0·04	0·2 ± 0·05 (9)
Volatile fatty acids		0·91 ± 0·09	0·82 ± 0·05	0·68 ± 0·09	0·68 ± 0·05	A 1·43 ± 0·1 } (6) V 0·36 ± 0·08 }
Protein (g/100 ml.)		2·47 ± 0·035	3·18 ± 0·14	2·4 ± 0·15	1·88 ± 0·07	7·55 ± 0·2 (9)
Glucose (mg/100 ml.)		59 ± 2·7	82 ± 3·8	82 ± 4·0	53 ± 0·75	A 61 ± 1·7 } (13) V 40 ± 1·0 }
Lymphocytes (cells/mm³)		17,500 ± 4·900	7,000 ± 650	2,930 ± 700	4,350 ± 1,100	—
α-amino N (mg/100 ml.)		—	4·09 ± 0·35 (4)	4·42 ± 0·5 (3)	3·12 ± 0·4 (8)	A 5·0 ± 0·31 } (6) V 3·39 ± 0·34 }
pH (38° C)		—	7·342 ± 0·015	—	—	A 7·40 ± 0·015 } (17) V 7·35 ± 0·015 }

Thus the figures recorded in pregnant animals are unreliable as estimates of mammary lymph flow, but the error in lactating non-pregnant goats is probably negligible. During lactation the skin and teat form only 20% of the total weight of the udder, which again is at least 40 times the weight



Text-fig. 3. Daily variations in milk yield, lymph flow and lymph protein from right mammary gland of goat Gracie. Cannulation on day 11, shown by arrows; the lymphatics from the vulva were resected in this animal on day 12. The fall in milk yield before cannulation occurred when the goat was moved from the farm to the animal house. It was associated with a lower rate of lymph flow and a higher lymph protein.

of the vulva at this stage because goats are anoestrous for the first 5–6 months of lactation and the vagina and vulva are small. In one goat (Gracie, Table 1 and Text-fig. 3), after the lymph duct was cannulated, 2 ml. Evans Blue (4%) solution was injected beside the vulva and the dye appeared in the cannula within 45 min. However, only about 36% of the

injected dye was recovered in the lymph, and when the perineal blood vessels and lymphatics were completely resected 26 hr later there was no significant change in the lymph flow.

Although there are blood vessels crossing the mid line between the two halves of the udder, there are no conspicuous lymphatics. The lymph flows were not significantly different in the two goats (Jill and Bessie, Table 1) in which the two glands of the udder had been previously permanently separated by a plastic skin operation.

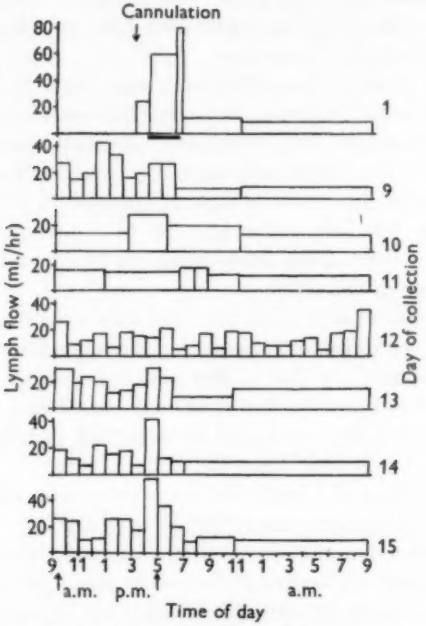
The daily loss of large quantities of lymph from these goats did not produce any noticeable ill effects. Adequate minerals were provided in the diet for milk production and pregnancy and water and salt licks were continuously available. There was no fall in plasma Na or Cl even after continuous collection for 17 days and no significant fall in milk yield in lactating animals. The losses of water, Na, Cl and protein were not great in relation to the weight of the animals (about 70 kg) and were usually less than the daily loss in the milk.

It will be seen from Table 1 and Text-fig. 2 that the average daily lymph flow varied with the daily milk yield. A similar relationship was also seen in individual animals from day to day and the clearest case is shown in Text-fig. 3. Since it has been found already that blood flow is related to milk yield (Linzell, 1960), as might be expected, lymph flow also varied with the average blood flow (Text-fig. 2).

The daily variations in lymph flow were not great, the standard deviation being about 10 % of the mean. In one goat the flow increased 50 % on the second day after cannulation when the operation wound was unusually oedematous. Hourly variations in flow were much greater (up to 300 %). Hourly collections for 33 hr in the goat Pam (Text-fig. 4) showed a standard deviation of 46 % of the mean. Although the rate of flow was sometimes highest at about the time the goats were milked and fed (9.30 a.m. and 5.0 p.m.) and was usually lowest in periods when the animals were mostly lying down (as at night and during the week-ends), at other times variations appeared to be random (Text-fig. 4). A marked increase in lymph flow (300–500 %) was produced when the animals' hind quarters were suspended in a sling during the recovery from epidural anaesthesia, presumably due to a rise in pressure in the abdominal veins produced in this way (Text-fig. 4). In one goat lymph flow was measured during pregnancy at the end of lactation and milking was stopped on the second day of lymph collection. There was no change in lymph flow either when the udder became distended with milk 6 days later or when this was relieved by milking out.

Lymph composition. A general analysis of the lymph collected daily is given in Table 1, together with the average composition of plasma from lactating Saanen goats of the same herd, collected over several years in

the course of other work. Variations in the composition of lymph collected hourly, which was investigated only in the case of Ca, Mg, P and glucose, were of the same order as the daily variations. However, variations in glucose content as high as 30 % of the mean were encountered in successive 5 min samples.



Text-fig. 4. Record of hourly lymph flow from right mammary gland on 8 days in the goat Pam. The animal was suspended in a sling to recover from the epidural spinal anaesthetic on day 1 as signalled by black bar and the lymph flow rose. The goat was milked each day at 9.30 a.m. and 5 p.m., shown by arrows.

As has been reported for lymph from some other organs (Yoffey & Courtice, 1956) the protein concentration tended to be higher at the lower rates of flow (Text-figs. 2, 3). As a result, the daily loss of protein in the lymph for the goats in Table 1, which were all in full lactation, was fairly constant (10·2, 11·3, 11·7 and 9·8 g/day). Electrophoretic analyses, by Dr A. E. Pierce of this Institute, of serum and lymph proteins collected simultaneously from the goat Jill (Table 1) showed no significant difference between arterial and mammary venous serum proteins, which consisted of 44·5 % albumin, 25·7 % α-globulin, 6·8 % β-globulin, and 23 % γ-globulin. By contrast, the lymph protein consisted of 55·2 % albumin, 21·8 % α-globulin, 7·1 % β-globulin and 15·9 % γ-globulin. There was also

probably fibrinogen in the lymph, because a fibrin-like material came down when heparinized lymph was thawed out after storage at $-10°$ C.

The differences between lymph and plasma shown in Table 1 are similar to those reported for other organs. Since the samples were not collected at the same time from the same goats the differences have not been tested for significance. However, in addition to these analyses, samples of arterial and mammary venous plasma (4 samples of each, collected every 2 hr and pooled) were compared with the lymph produced simultaneously (during 8 hr) in the goat Bessie. The analyses were very similar to the figures in Table 1; lymph Cl, K and HCO_3 were all 5% higher than the venous plasma values, Ca and Mg 18·5 and 19% lower and Na and inorganic P not significantly different. Lymph glucose and VFA, which are actively taken up from the blood by the lactating mammary gland and were identical to the average figures for this animal in Table 1, were midway between arterial and venous plasma levels. In other goats the lymph concentrations of glucose and VFA were nearer the venous plasma figures.

Lymph flow in anaesthetized animals

Shorter collections ($\frac{1}{2}$–4 hr) were also made in 15 goats, 2 sheep and a dog under general anaesthesia (cyclopropane in goats and sheep, and pentobarbitone in the dog) mostly during the course of other experiments involving dissection around the mammary glands, which often reduces both the blood flow and rate of milk secretion (Linzell, 1960 and unpublished). Although the milk yields of the goats before the experiments were in the same range as those in Table 1, the rates of lymph flow during the experiments were much less than in the conscious animals (0·1–1·25 ml./100 g/hr) and the average protein concentration was significantly higher ($4{\cdot}41 \pm 0{\cdot}35$ g/100 ml.). A smaller number of analyses showed raised levels of Ca (2·2–4·5 m-equiv/l.), Mg (3·3 and 3·8 m-equiv/l.), inorganic P (3–7·4 m-equiv/l.) and glucose (36–156 mg/100 ml.). In two experiments lymph K and lactate were measured at the start of perfusion of the isolated mammary gland of the goat (time between removal from the anaesthetized animal and starting the perfusion 5 and 10 min). K was initially raised to 5 m-equiv/l. and lactate to 0·9 and 1·2 m-equiv/l., but these quickly fell once perfusion had started to 4·6 m-equiv/l. for K and 0·14 m-equiv/l. for lactate. Lymph flows from one mammary gland of the anaesthetized sheep were 1 and 2 ml./100 g/hr and in the dog about 4 ml./100 g/hr.

DISCUSSION

Mammary lymph has been collected in this laboratory for two reasons. First, to assess the variable rates of lymph flow encountered in perfused goats' udders (Cargill-Thompson *et al.* 1958; Hardwick *et al.* 1960) and

secondly to determine the proportion of potential milk precursors that are lost from the mammary glands in the lymph in conscious animals. Having measured the uptake quantitatively by simultaneous measurement of blood flow and arteriovenous differences (Linzell, 1960) it seemed necessary to know the quantities removed as lymph, since several workers have previously pointed out that this is an unmeasured source of error in work of this sort.

In answer to the first problem it seems that the rate of lymph flow reflects the general metabolic activity of the tissue, running roughly parallel to blood flow and to the milk yield of each gram of tissue, and is thus one means of investigating the condition of glands being perfused. As to the second problem, it is clear that only a small proportion of milk precursors removed from the plasma return to the circulation via the lymph. In many studies this factor may be ignored because the quantities in lymph are small in relation to the errors in measuring the mean uptake from the plasma (because of the high blood flow the arteriovenous differences are often small). It has been calculated from the present data and those from Linzell (1960) that only 2% of the Ca removed from the plasma appears in the lymph and about 0·5% of the glucose.

One of the unexpected features of this work was the high rate of lymph flow from the mammary gland, which in conscious lactating animals is about 10 times the average flow from the thoracic duct in other animals (Yoffey & Courtice, 1956) and about equal to the flow of lymph from the liver in unanaesthetized dogs (Ritchie, Grindlay & Bollman, 1959) and rats (Friedman, Byers & Omoto, 1956). Unlike that from the liver, however, the protein content at the higher rates of flow is low and is inversely related to the flow. Mammary lymph flows spontaneously not only in conscious resting animals, but in anaesthetized animals and in perfused mammary glands. The rate of flow, which has been found to be related to the rates of milk secretion and blood flow per unit volume of tissue at different stages of lactation, is ordinarily, like the milk yield and blood flow (Linzell, 1960) fairly constant in conscious animals. The greater variation encountered from hour to hour than from day to day may merely reflect the greater scatter of observations to be expected when a randomly varying process is sampled more frequently. The fact that the rate of lymph flow was not clearly related to milking (when the udder is massaged) or to the state of fullness with milk, conditions under which venous pressure variations might be expected, was surprising. However, it may be said that this is testimony to the remarkable adaptation of the udder of domesticated milking ruminants, that can hold and release the large quantities of milk that accumulates between milkings (up to 4 l. in the goat) whilst maintaining a constant over-all rate of metabolism.

The high rates of lymph flow during pregnancy were also surprising, and, as has already been pointed out, the amount of lymph coming from the vulva must partly account for this increase. Nevertheless, the mammary gland during pregnancy in these goats was considerably larger than all the perineal tissues and it seems likely that there was an increase in mammary lymph flow as well. However, there is the possibility that this could be due to a raised venous pressure associated with pregnancy. It has been demonstrated that a raised abdominal pressure, as produced by supporting the animal in an abdominal sling, greatly increased the lymph flow. One of the main veins draining the udder passes into the abdomen, through the inguinal canal, where it would be subjected to raised average abdominal pressure and also could occasionally be mechanically compressed by the foetus.

In general composition mammary lymph is similar to that from other organs. According to the classification of Yoffey & Courtice (1956) the lymph collected in this work is intermediate (i.e. lymph that has passed through one lymph gland only), as distinct from peripheral and central lymph. The numbers of lymphocytes are in agreement with the numbers in intermediate lymph from other regions. As would be expected according to the generally accepted theory of lymph formation, mammary lymph is lower than the plasma in Na plus K, Ca and sometimes Mg, but higher in Cl and slightly higher in HCO_3. In addition there is significantly less protein, but a greater proportion of those of smaller molecular weight (e.g. albumin). In conscious animals during lactation the concentration of those substances (glucose, VFA, amino acids) actively utilized by the mammary tissue lies between arterial and venous levels, but in non-lactating or anaesthetized animals, when the uptake is reduced, the levels approach arterial values as is the case with non-electrolytes in lymph generally.

The lymph flow during acute experiments under general anaesthesia in goats was lower than in conscious animals and there were in addition significant changes in lymph composition. These changes may well be due to the reduced blood flow following dissection around the mammary glands in these experiments, but general anaesthesia itself may lower the mammary blood flow and a lower lymph flow was also encountered in one experiment where only lymph cannulation was done with general anaesthesia.

It must be pointed out that there are some significant differences between the composition of the lymph collected in this work from conscious goats and that taken from dead cows by Heyndrickx & Peeters (1958*a*, *b*, 1960) and Heyndrickx (1959). There is some evidence to suggest that many of the differences may be due to post-mortem changes. The Belgian workers themselves point out that Na and K were unexpectedly higher in the

lymph collected after death than in plasma taken before slaughter and stress that their lymph had an extremely high level of lactate (about 10 m-equiv/l.). Comparison of the present figures with their published findings shows the following additional differences. In the goat lymph HCO_3 is slightly *higher* than in the plasma, and lymph inorganic P, Mg and amino acids are slightly *lower* than in plasma. Some of these changes occurred in lymph collected during acute experiments under general anaesthesia when the rate of lymph flow was also reduced, possibly associated with a reduced blood flow and relative anoxia. However, the most striking differences are that the K and lactate in dead cow udder lymph are 2 and 12 times higher than in that from conscious goats. Both these changes would be expected in anoxia and indeed were seen to a lesser degree when goats' udders (cooled during dissection) were removed for perfusion, under epidural anaesthesia. This suggests that the recent findings of Heyndrickx & Peeters (1960), that the lymph content of lactose, citrate and α-oxoglutarate are higher than in plasma, may also be due to post-mortem anoxic changes.

Although it may be that a knowledge of the variations in the quantities of metabolites in mammary lymph at different stages of lactation will provide clues to the type of metabolism going on at the time, it would seem necessary to interpret with caution the results of analyses of lymph taken from dead or anaesthetized animals.

SUMMARY

1. A method is described for the continuous collection of mammary gland lymph in unrestrained conscious goats.

2. Lymph was collected for up to 17 days at the rate of 150–840 ml./day from one mammary gland without disturbing the animal.

3. Mammary lymph flows spontaneously at a rate, which varies with the rate of milk secretion and the mean blood flow, from 0·3 to 2·71 ml./100 g/hr. Daily variations in flow were 10% (S.D.) of the mean but the hourly variations averaged 46%. The protein concentration varies inversely with the rate of flow.

4. The composition of the lymph and its relationship to plasma are similar to those of lymph from other organs.

5. Lymph flow was reduced in experiments under general anaesthesia and the composition altered. This is discussed in relation to the high K and lactate recorded by Heyndrickx & Peeters (1958*a*) in the udder lymph from dead cows.

I wish to thank Dr A. E. Pierce for doing the electrophoretic analyses of proteins, Dr D. C. Hardwick and Miss S. M. Price for help with some of the analyses and I. R. Fleet and R. D. Burton for technical assistance.

REFERENCES

ANNISON, E. F. (1954). Studies on the volatile fatty acids of sheep blood with special reference to formic acid. *Biochem. J.* **58**, 670–680.

BARKER, J. N. & BRITTON, H. G. (1957). The enzymatic estimation of l(+)-lactic acid. *J. Physiol.* **138**, 3*P*–4*P*.

BERENBLUM, I. & CHAIN, E. (1938). An improved method for the colorimetric determination of phosphate. *Biochem. J.* **32**, 295–298.

CARGILL-THOMPSON, H. E. C., DRURY, A. N., HARDWICK, D. C., LINZELL, J. L. & TUCKER, E. M. (1958). The blood flow and milk yield of goats' udders perfused with artificial media. *J. Physiol.* **143**, 74–75*P*.

FRIEDMAN, M., BYERS, S. O. & OMOTO, C. (1956). Some characteristics of hepatic lymph in the intact rat. *Amer. J. Physiol.* **184**, 11–17.

HAMILTON, P. B. & VAN SLYKE, D. D. (1943). The gasometric determination of free amino-acids in blood filtrates by the ninhydrin-carbon dioxide method. *J. biol. Chem.* **150**, 231–250.

HARDWICK, D. C., LINZELL, J. L. & PRICE, S. M. (1960). The rate of incorporation of ^{32}P into milk and casein by the perfused udder. *Biochem. J.* **75**, 5*P*.

HEYNDRICKX, G. V. (1959). Investigations on the lipids, proteins, lipo- and glycoproteins of udder lymph and plasma in cattle. *Quart. J. exp. Physiol.* **44**, 264–270.

HEYNDRICKX, G. V. & PEETERS, G. (1958*a*). Investigations on the ionic balance of udder lymph among cattle. *Quart. J. exp. Physiol.* **43**, 174–179.

HEYNDRICKX, G. V. & PEETERS, G. (1958*b*). Investigations on the enzymes in udder lymph, plasma and milk of cattle. *Enzymologia*, **20**, 161–166.

HEYNDRICKX, G. V. & PEETERS, G. (1960). Concentration of sugars, ketone bodies and some organic acids in udder lymph and plasma of cattle. *Biochem. J.* **75**, 1–3.

LINZELL, J. L. (1959). The innervation of the mammary glands in the sheep and goat with some observations on the lumbo-sacral autonomic nerves. *Quart. J. exp. Physiol.* **154**, 160–176.

LINZELL, J. L. (1960). Mammary-gland blood flow and oxygen, glucose and volatile fatty acid uptake in the conscious goat. *J. Physiol.* **153**, 492–509.

NELSON, N. (1944). A photometric adaptation of the Somogyi method for the determination of glucose. *J. biol. Chem.* **153**, 375–380.

RITCHIE, H. D., GRINDLAY, J. H. & BOLLMAN, J. L. (1959). Flow of lymph from canine liver. *Amer. J. Physiol.* **196**, 105–109.

SOMOGYI, M. (1952). Notes on sugar determination. *J. biol. Chem.* **195**, 19–23.

VAN SLYKE, D. D. & HILLER, A. (1947). Application of Sendroy's iodometric chloride titration to protein-containing fluids. *J. biol. Chem.* **167**, 107–124.

VAN SLYKE, D. D. & SENDROY, J. (1928). Line charts for graphic calculations by the Henderson–Hasselbalch equation, and for calculating plasma carbon dioxide content from whole blood content. *J. biol. Chem.* **79**, 781–798.

WILSON, A. A. (1955). The determination of magnesium and calcium in serum or plasma with ethylene diaminetetra-acetic acid (EDTA). *J. comp. Path.* **65**, 285–290.

YOFFEY, J. M. & COURTICE, F. C. (1956). *Lymphatics, Lymph and Lymphoid Tissue*, 2nd ed. London: Edward Arnold.

J. Physiol. (1960), **153**, *pp.* 522–526
With 3 plates
Printed in Great Britain

MOTOR INNERVATION OF EXTRAOCULAR MUSCLE

By C. KUPFER

From the Howe Laboratory of Ophthalmology, Harvard Medical School Boston, Massachusetts, U.S.A.

(*Received* 18 *May* 1960)

There has been considerable interest recently in the motor innervation of skeletal muscle, particularly with respect to multiple innervation of single muscle fibres. For many muscles there exists clear histological and physiological evidence that individual fibres possess more than one end-plate (Feindel, Hinshaw & Weddell, 1952; Hunt & Kuffler, 1954). For many of these muscle fibres the innervation is polyneuronal, i.e. the end-plates are derived from different axons (Hunt & Kuffler, 1954). Regarding the muscle spindles, there is good evidence of polyneuronal innervation by small-diameter motor nerves which go exclusively to intrafusal fibres (Hunt & Kuffler, 1951). Larger-diameter motor nerves also innervating spindles have been suggested by histologists (Copper & Daniel, 1956; Boyd, 1958) and more recently by Granit, Pompeiano & Waltman (1959).

The present study is an investigation of the motor nerve innervation of human extraocular muscle by means of the histochemical localization of cholinesterase (ChE). Since ChE is localized at sites of acetylcholine release at motor nerve endings in striated muscle (Koelle, 1951) the pattern of ChE localization will indicate motor nerve innervation of the extrafusal and intrafusal muscle fibres. Extraocular muscle is ideally suited for such a study. Each muscle is relatively short (40 mm), easily available and has been studied recently in some detail (Cooper & Daniel, 1949; Wolter, 1954; Cooper, Daniel & Whitteridge, 1955). Each extrafusal muscle fibre extends the entire length of the muscle (Brown & Harvey, 1941). There are many muscle spindles, most numerous at the proximal end. In the region of the motor nerve endings on extrafusal fibres, there are few muscle spindle efferent endings (Cooper & Daniel, 1949).

METHODS

Human extraocular muscle was obtained from the operating room. Fresh autopsy material was used when available. The complete muscle was placed in cold 10% neutral formalin for 6 hr after which frozen sections were cut 25–50 μ thick. These were placed in Coërs modification of Koelle's incubation solution (pH 5) for 15–45 min (Coërs & Woolf, 1959). The incubation solution contains copper glycine and the substrate acetylthiocholine. The

latter compound is hydrolysed by the ChE localized in the tissue and copper thiocholine is precipitated at these sites of enzyme action. This white precipitate is converted to black copper sulphide by treating the tissue sections with ammonium sulphide solution. The sections are washed, dehydrated and mounted. The photomicrographs are taken through crossed polaroid prisms unless noted otherwise.

RESULTS

Fourteen muscles were examined including at least one specimen of each of the six extraocular muscles. There did not appear to be any qualitative differences in the sites of ChE activity among the six extraocular muscles. Consequently, the following description applies to all the muscles studied.

Extrafusal muscle fibres. A broad band of ChE activity was localized in the middle two-thirds of the muscle. The typical configuration of the subneural apparatus of the motor end-plate (terminaisons en plaque) was apparent (Pl. 1, fig. 1). From two-thirds to three-quarters of the muscle fibres possessed two or more end-plates (Pl. 1, figs. 2–4). It was not possible to determine whether the multiple end-plates were from the same or different nerve fibres i.e. whether the innervation was polyneuronal. About 5–10% of the motor end-plates were outside the main band but owing to the fragmentation of the muscle fibres in sectioning the tissue, one could not determine whether or not these end-plates were on muscle fibres already innervated.

On a number of fibres an additional small site of ChE activity was noted adjacent to the main end-plate configuration (Pl. 1, figs. 5, 6). These small sites did not appear to be part of the end-plate for they lacked the typical subneural apparatus structure, being only a discrete stained area. These sites were either single or at most double near an end-plate.

At the musculo-tendinous junction at either end of the muscle the extrafusal fibre tip stained for the presence of ChE activity (Pl. 2, figs. 7–10), this being the so-called cholinesterasic cuffs described by Couteaux (1953) in skeletal muscle.

Muscle spindle. The muscle spindles were located in the proximal and distal portions of the muscle, being more abundant in the former. The ChE localized in the spindles stopped short of the band of end-plates and then continued on the other side toward, but not reaching the distal end of the muscle. There were two patterns of ChE localization:

(1) Thin intrafusal fibres with discrete loci of ChE activity scattered along the entire length occupying peripheral sites on the fibre. In some sections, the entire fibre was seen and these were the only sites of ChE activity (Pl. 3, figs. 11–13).

(2) Medium-sized intrafusal fibres containing both typical (albeit) small motor end-plates with subneural apparatus and the small discrete loci of

ChE activity (Pl. 3, fig. 14). These fibres were not as plentiful as the thin fibres, but were usually located in close proximity to the latter (Pl. 3, figs. 15, 16).

DISCUSSION

Motor innervation of the extrafusal fibres. Although polyneuronal innervation has been demonstrated in the cricothyroideus, soleus and gastrocnemius muscles of the cat (Hunt & Kuffler, 1954), there is no evidence that the multiple end-plates on extraocular muscle fibres are derived from more than one axon. On the contrary, Feindel *et al.* (1952), describing the pattern of innervatiou of the extraocular muscles of the rabbit and macaque monkey, noted a single nerve fibre giving off multiple endings along a considerable length of one muscle fibre. This arrangement appears to be unique to the extraocular muscles, not being seen in other muscles so far examined.

Multiple innervation (as opposed to polyneuronal innervation) may influence the rate at which tension is developed in a muscle fibre. The twitch tension of cat extraocular muscle reaches its maximum within 7·5 msec (Cooper & Eccles, 1930; Hunt, unpublished observation) which is much more rapid than any other muscle examined. If nerve impulses reach endings synchronously at several sites along a muscle fibre, the contractile system would be activated simultaneously over a large portion of the muscle fibre. With simultaneous activation of almost the entire muscle fibre, tension would develop more rapidly and account for such a short contraction time.

The significance of small sites of ChE activity adjacent to the motor end-plate on extrafusal fibres is difficult to assess on a purely anatomical basis. Wolter (1954) described '. . . vegetative nerves with loops in or outside the motor plate proper'. He considered such endings parasympathetic nerves since they ran parallel with branches of the cranial nerves. In the adrenal and parotid glands, as well as in the intestine, ChE has been localized near post-ganglionic parasympathetic nerve endings (Koelle, 1951). Whether the small sites of ChE activity represent this type of ending must await further work.

The cholinesterasic cuffs of muscle fibres at the musculo-tendinous junction has been described by Couteaux (1953) for skeletal muscle. The significance of the enzyme activity at this location is unknown.

Motor innervation of the muscle spindle. It is generally accepted that mammalian muscle spindles are innervated by gamma efferent nerve fibres (Kuffler, Hunt & Quilliam, 1951) and more than one gamma efferent nerve may terminate on the same intrafusal fibre (Barker, 1948; Hunt & Kuffler, 1951). In addition to the gamma efferent nerves, there is evidence to suggest

that other efferent nerves of larger diameter than gamma efferents (Cooper & Daniel, 1956; Boyd, 1959) having faster conduction than that of gamma efferents (Granit *et al.* 1959; Diete-Spiff & Pascoe, 1959) also innervate muscle spindles. These efferent nerves may be alpha nerve fibres. On the basis of ChE localization, there are two types of motor nerve endings in the muscle spindles of skeletal muscle (Coërs & Woolf, 1959) and in human extraocular muscle spindles. The thin intrafusal muscle fibres having the ChE activity localized in discrete loci along the fibre may be innervated by gamma efferent nerves only. The larger intrafusal fibres having ChE activity localized at both typical end-plates and discrete loci may be innervated by both alpha and gamma efferent nerves, respectively. This would agree with Boyd's classification of simple and compound muscle spindles in cat skeletal muscle (1959) and Cooper, Daniel & Whitteridge's description of the human eye muscle spindle (1955).

SUMMARY

1. Histochemical localization of the enzyme cholinesterase (ChE) was utilized in studying the motor nerve innervation of human extraocular muscle.

2. Multiple motor end-plates on extrafusal fibres were demonstrated and their significance discussed. In addition, smaller sites of ChE activity near the motor end-plate were described. The possibility of these sites representing post-ganglionic parasympathetic efferent nerve endings was mentioned. Cholinesterasic cuffing at the ends of the extrafusal fibres at the musculo-tendinous junction was also noted.

3. On the basis of the ChE localization in muscle spindles, there appear to be two types of motor nerve endings. It is suggested that both the alpha and gamma efferent nerve fibres can innervate some intrafusal fibres while others are innervated by gamma efferent nerves only.

REFERENCES

Barker, D. (1948). The innervation of the muscle-spindle. *Quart. J. micr. Sci.* **89**, 143–186.

Boyd, I. A. (1958). The innervation of mammalian neuromuscular spindles. *J. Physiol.* **140**, 14*P*.

Boyd, I. A. (1959). Simple and compound mammalian muscle spindles. *J. Physiol.* **145**, 55–56*P*.

Brown, G. L. & Harvey, A. M. (1941). Neuromuscular transmission in the extrinsic muscles of the eye. *J. Physiol.* **99**, 379–399.

Coërs, C. & Woolf, A. L. (1959). *The Innervation of Muscle*, 1st ed. Springfield: Charles C. Thomas.

Cooper, S. & Daniel, P. M. (1949). Muscle spindles in human extrinsic eye muscles. *Brain*, **72**, 1–24.

Cooper, S. & Daniel, P. M. (1956). Human muscle spindles. *J. Physiol.* **133**, 1*P*.

Cooper, S., Daniel, P. M. & Whitteridge, D. (1955). Muscle spindles and other sensory endings in the extrinsic eye muscles; the physiology and anatomy of these receptors and of their connections with the brain stem. *Brain*, **78**, 564–583.

COOPER, S. & ECCLES, J. C. (1930). The isometric responses of mammalian muscles. *J. Physiol.* **69**, 377–385.

COUTEAUX, M. R. (1953). Particularités histochimiques des zones d'insertion du muscle strié. *C.R. Soc. Biol., Paris*, **147**, 1974–1976.

DIETE-SPIFF, K. & PASCOE, J. E. (1959). The spindle motor nerves to the gastrocnemius muscle of the rabbit. *J. Physiol.* **149**, 120–134.

FEINDEL, W., HINSHAW, J. R. & WEDDELL, G. (1952). The pattern of motor innervation in mammalian striated muscle. *J. Anat., Lond.*, **86**, 35–48.

GRANIT, R., POMPEIANO, O. & WALTMAN, B. (1959). Fast supraspinal control of mammalian muscle spindles: extra- and intrafusal co-activation. *J. Physiol.* **147**, 385–398.

HUNT, C. C. & KUFFLER, S. W. (1951). Further study of efferent small-nerve fibres to mammalian muscle spindles. Multiple spindle innervation and activity during contraction. *J. Physiol.* **113**, 283–297.

HUNT, C. C. & KUFFLER, S. W. (1954). Motor innervation of skeletal muscle: Multiple innervation of individual muscle fibres and motor unit function. *J. Physiol.* **126**, 293–303.

KOELLE, G. B. (1951). The elimination of enzymatic diffusion artifacts in the histochemical localization of cholinesterases and a survey of their cellular distributions. *J. Pharmacol.* **103**, 153–171.

KUFFLER, S. W., HUNT, C. C. & QUILLIAM, J. P. (1951). Function of medullated small nerve fibres in mammalian ventral roots; efferent muscle spindle innervation. *J. Neurophysiol.* **14**, 29–54.

WOLTER, J. R. (1954). The morphology of the nervous system of the striated muscles of the human eye. *Acta XVIII Conc. Ophth.* 1865–1873.

EXPLANATION OF PLATES

All photomicrographs are from human extraocular muscle stained for the presence of cholinesterase (ChE) activity. Crossed polaroid prisms were used for all photomicrographs except for Figs. 11, 12 and 15 of Pl. 3. All scales = 100 μ.

PLATE 1

Longitudinal sections of extrafusal muscles fibres showing the localization of ChE at the motor end-plates and in adjacent small loci. (MEP, motor end-plate; L, small locus of ChE activity on extrafusal muscle fibre).

Fig. 1. Motor end-plates with the subneural apparatus containing the ChE.

Figs. 2–4. Multiple motor end-plates on individual extrafusal muscle fibres.

Figs. 5, 6. A small locus of ChE adjacent to an end-plate on the extrafusal muscle fibres.

PLATE 2

Longitudinal sections of the musculo-tendinous junction showing the cholinesterasic cuffs on the ends of the extrafusal muscle fibres. (T, tendon; M, muscle.)

PLATE 3

Longitudinal sections (except Fig. 15) of muscle spindles showing the two types of ChE localization on intrafusal muscle fibres. (MEP, motor end-plate; l: small loci of ChE on intrafusal muscle fibres).

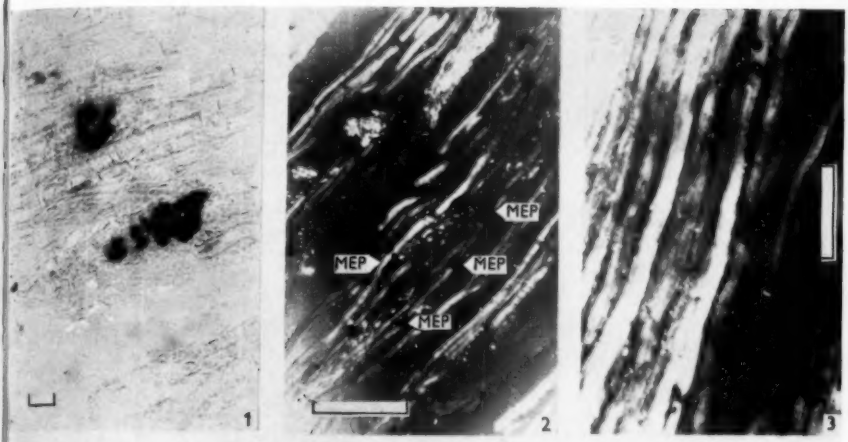
Figs. 11, 12. Small loci of ChE scattered along the intrafusal muscle fibres.

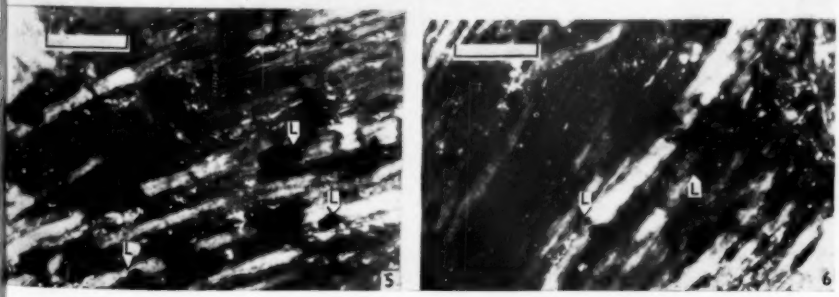
Fig. 13. The entire intrafusal muscle fibre with small loci of ChE is shown between the two arrows.

Fig. 14. A muscle spindle with typical motor end-plates and small loci of ChE on adjacent intrafusal muscle fibres.

Fig. 15. A teased preparation of a muscle spindle showing an intrafusal muscle fibre with both typical motor end-plates and small loci of ChE.

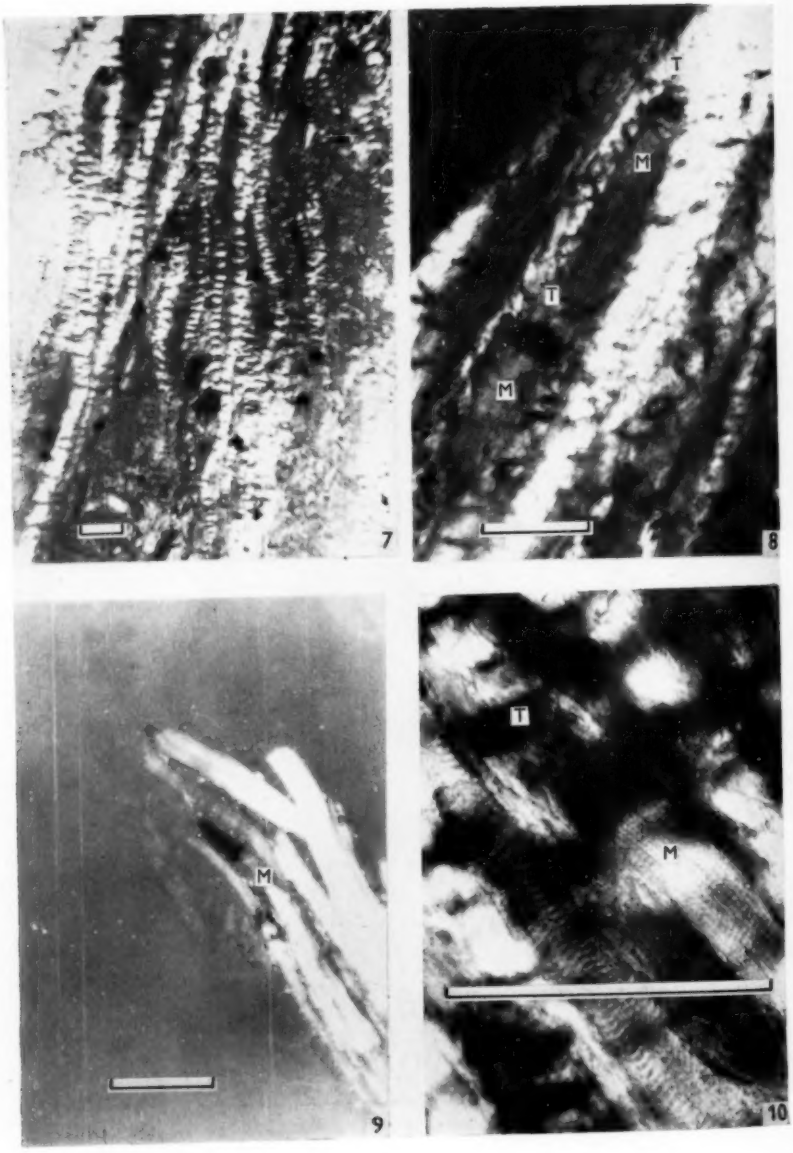
Fig. 16. Motor end-plates and small loci of ChE appear on the same intrafusal muscle fibre.





C. KUPFER

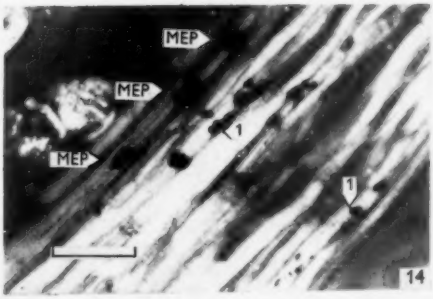
(*Facing p.* 526)

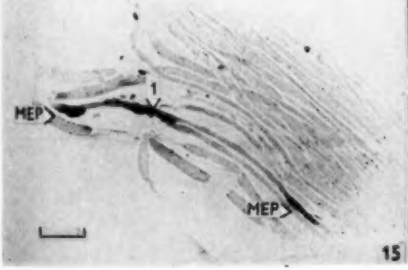

7
T
M
T
M
8
M
9
T
M
10

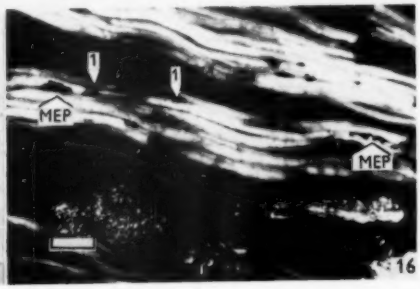
11

12

13


MEP
MEP
MEP
1
1
14


MEP
1
MEP
15


1
1
MEP
MEP
16

J. Physiol. (1960), **153**, *pp.* 527–552
With 7 text-figures
Printed in Great Britain

BLOOD SUGARS AND TISSUE CARBOHYDRATE IN FOETAL AND INFANT LAMBS AND RHESUS MONKEYS

By HEATHER J. SHELLEY

From the Nuffield Institute for Medical Research, University of Oxford and the National Institute of Neurological Diseases and Blindness, Field Station of Perinatal Physiology, San Juan, Puerto Rico

(*Received* 18 *May* 1960)

During a previous investigation (Dawes, Mott & Shelley, 1959), the carbohydrate content of various tissues from foetal lambs just over halfway through gestation was determined for comparison with that of foetuses near term. The carbohydrate concentrations in the heart, liver, lungs and skeletal muscle were very different in the two groups, and the work has now been extended to include lambs of earlier and intermediate ages so as to follow the changes in carbohydrate concentration throughout the last two-thirds of gestation. Large amounts of glycogen were laid down towards the end of gestation, not only in the liver but also in skeletal muscle, and measurements of tissue carbohydrate have been made in new-born and infant lambs of up to 17 days old so as to determine its fate after birth. The lamb is relatively mature at birth and exceptionally active, and the results suggest that both the liver and the skeletal muscle carbohydrate constitute carbohydrate reserves for use in the first 24 hr of extrauterine life. The rhesus monkey is also fairly mature at birth, and estimates of tissue carbohydrate have been obtained in this species during the last third of gestation and in infant monkeys 8–15 days old. This paper compares the results obtained on lambs and monkeys with those in other species and discusses their possible significance. A preliminary account of this work has already been published (Shelley, 1960).

METHODS

Experimental procedure and animals

Foetal lambs. Observations have been made on forty-six foetal lambs (gestation age 58–146 days) from twenty-four cross-bred ewes; eight ewes carried single lambs, eleven carried twins, four carried triplets and one ewe carried quadruplets and developed signs of pregnancy toxaemia a few days before the experiment.

The lambs were delivered by Caesarean section under chloralose or pentobarbitone anaesthesia and were left attached to the ewe, care being taken to avoid stretching or compressing the umbilical cord. A foetal blood sample (0·8 ml.) was taken into a 1 ml. syringe, the dead space of which had been filled with 0·4% heparin in sodium fluoride

solution 8% (w/v), either from an arterial catheter or from the heart. A maternal blood sample had usually been taken a few minutes earlier by direct jugular puncture before anaesthesia or from a catheter in a forelimb artery after beginning anaesthesia. Foetal tissue samples (100–200 mg) were taken as soon as possible after delivery. In nine experiments the order of sampling was: skeletal muscle (lateral aspect of thigh), liver, lung (apex of left upper lobe), heart (apex of ventricles), cerebral cortex and kidney cortex. Brain and kidney samples were not taken from the remaining foetuses and in ten experiments the order of sampling was varied; the time interval between sampling different tissues did not exceed 2 min and the order of sampling did not appear to affect the results, though, since cardiac glycogen is very readily depleted in anoxia, it was considered advisable to sample the heart first. Samples of one or two tissues only were taken from each of another eleven lambs and blood samples only from the remaining sixteen since these were later asphyxiated by tying the umbilical cord and could not then be regarded as normal material (Dawes *et al.* 1959). In eight experiments blood and tissue samples were not obtained until at least one sibling had been asphyxiated, but during this period (1–2 hr) the lamb had remained *in utero*.

At the end of each experiment the foetus was weighed. Individual organs, including total skeletal muscle, were also weighed in nine experiments on lambs of 85–138 days gestation age; the results obtained, when expressed as percentage of body weight, were similar to those of Carlyle (1948) and Wallace (1948) and their data were therefore used to calculate the organ weights in the remaining foetuses.

Infant lambs. Venous blood and tissue samples were obtained from six lambs 2½–24 hr after normal delivery and from nine lambs 5–17 days old, all under pentobarbitone anaesthesia. Individual organs and total skeletal muscle were weighed in all experiments but one. Blood samples and skeletal muscle (usually semitendinosus) only were also taken from seven lambs under chloralose anaesthesia and from three lambs under local anaesthesia (xylocaine) at ages varying from 3 hr to 12 days after birth. In three other experiments muscle and blood samples were taken from the same lamb at different ages under local anaesthesia, final samples being obtained under chloralose anaesthesia. The lambs included four singletons, two from different sets of twins, seven complete sets of twins, two from one set of triplets and two complete sets of triplets. They had been housed in unheated premises with their dams up to the day of the last sample and were usually weighed on the day of the experiment.

Monkeys. Blood and tissue samples were obtained from twelve foetal rhesus monkeys (gestation age 115–158 days) and from three of their mothers, from seven infant monkeys (8–15 days old) hand-reared at 30° C and from two adolescent monkeys (1–2 years old), all under pentobarbitone anaesthesia. Blood samples were obtained from the foetal and six of the infant monkeys 1–2 hr after anaesthesia and tissue samples after 2–4 hr anaesthesia; these monkeys were being used for other studies (Dawes, Jacobson, Mott & Shelley, 1960), hence the delay. However, in all twelve foetal experiments the blood samples were taken while the foetus was still *in utero* (either from a leg withdrawn through a small uterine incision or from a carotid artery dissected *in utero*). In seven experiments the foetus was retained *in utero* throughout, and the tissues were sampled immediately after delivery by Caesarean section, while the foetus was still in good condition and attached to the mother by the umbilical cord; in one experiment the tissues were sampled 30 min after delivery but with the cord still intact, and in four others the foetuses (116–145 days gestation age) were deliberately asphyxiated by clamping and dividing the umbilical cord after delivery. Samples were also obtained, without anaesthetic, from a thirteenth foetus (gestation age 117 days) which had been born vaginally in a partially asphyxiated state. Two of the infant monkeys had been made anoxic by giving them 100% nitrogen to breathe and one infant was apnoeic for about 2 min shortly before the tissues were sampled. The adult monkey tissues were sampled after 4–5 hr of anaesthesia but samples were obtained from the adolescent monkeys and the

15-day-old infant within a few minutes of inducing anaesthesia. All the monkeys were singletons.

The sites of tissue sampling were similar to those described for lambs, except that biceps muscle was taken from the vaginally-born foetal monkey. Blood samples were taken from arterial catheters in the foetuses and two infants, from venous catheters in two infants, direct from the short saphenous vein in the adults, and direct from the heart in the adolescents and oldest infant. Total body weight and organ weights (liver, lungs and heart) were obtained for all the foetal monkeys, six infants and two adults.

Biochemical and histological methods

Blood. 0·2–0·5 ml. was deproteinized with NaOH and $ZnSO_4$ and the extract (dilution 1 in 10) was used for the determination of glucose (Huggett & Nixon, 1957), fructose (Bacon & Bell, 1948) and lactate (Barker & Summerson, 1941), except that in the sheep experiments lactate determinations were made on trichloroacetic acid extracts of blood.

Tissues. The tissue samples were divided into two equal portions. Within 10–20 sec of removal one portion was placed in McClung's picric acid-dioxane fix No. 2 (Cowdry, 1948), and was subsequently stained for glycogen by Best's carmine method, and the other was placed in 1·0 ml. deproteinizing solution (5 % trichloroacetic acid containing Ag_2SO_4 200 mg/100 ml.) in a weighed glass receptacle. This was reweighed immediately and the tissue was then chopped up finely with scissors and homogenized in a Potter–Elvehjem homogenizer. The homogenate was diluted to 5 ml. with deproteinizing solution, heated for 15 min in a boiling water-bath, cooled and centrifuged. The volume of the supernatant was adjusted to 5 ml. and was used for the determination of total carbohydrate, i.e. glycogen + glucose + fructose (Kemp & Kits van Heijningen, 1954; Mendel, Kemp & Myers, 1954), fructose and lactate.

RESULTS

Term occurs at about the 147th day of gestation in sheep and the 168th day in rhesus monkeys. Thus there is comparatively little difference in the length of the gestation period, and in Figs. 1 and 3–7 the results have been plotted against the number of days before term rather than against gestational age, since this made possible a closer comparison between the species. Lactate determinations were made on most of the blood and tissue samples as a check on the degree of hypoxia produced by the experimental conditions.

Blood sugars

Glucose. In Fig. 1 the blood glucose concentration has been plotted against age for lambs (○) from 58 to 138 days gestation age (89–9 days before term) and 2½ hr to 17 days after birth, and for monkeys (●) from 115 to 158 days gestation age (53–10 days before term) and 8–15 days after birth. Figure 1 includes not only results from the unasphyxiated foetal lambs which were used for the present tissue-carbohydrate study but also those obtained under similar conditions from their siblings before these were asphyxiated (see Methods); the values shown for foetal and infant monkeys were also obtained before any were made anoxic. It can be seen that in general the foetal blood glucose level was lower in the lambs than

in the monkeys and that there was no change with increasing gestational age in either species, though there was a wide scatter in both species, 3–32 mg/100 ml. in the lambs and 20–36 mg/100 ml. in the monkeys. In the lambs this scatter existed among litter-mates and was partly associated with the order of delivery and the duration of the experiment, since, in thirteen of fifteen experiments on sheep with multiple pregnancies, the foetal blood glucose was lower in the second or third members of sets of twins or triplets than in that delivered first. This difference was slight, less

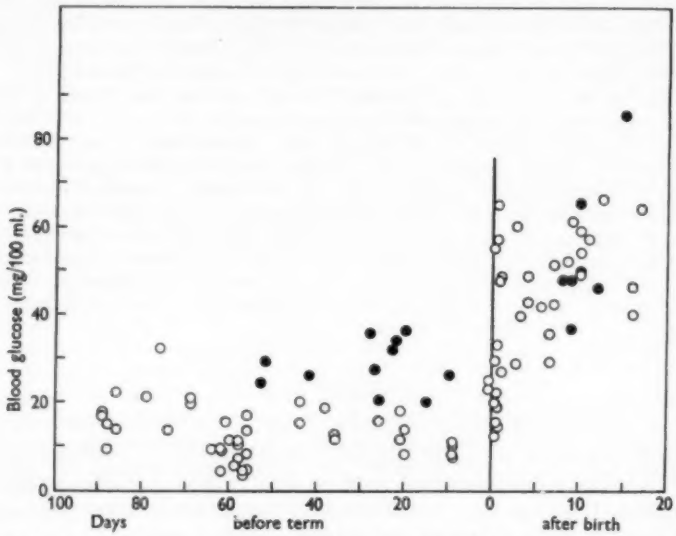


Fig. 1. Changes in blood glucose concentration with age in foetal and infant lambs (○) and monkeys (●). The results have been plotted against days before term for the foetuses and days after birth for the infants; the vertical line represents the time of birth.

than 2 mg/100 ml., when the foetuses were delivered within 20 min of each other but was sometimes considerable (5–10 mg/100 ml.) when the time interval was longer. The very low levels, less than 8 mg/100 ml., were all observed in foetuses from multiple pregnancies delivered after several hours anaesthesia.

Since it is known that anoxia affects the blood glucose level in foetal lambs and monkeys (Dawes *et al.* 1959; 1960), and since hypoxia is easy to produce unintentionally under these conditions, the lactate content of the foetal blood samples was considered in relation to their glucose content. Although, in the foetal monkeys, the highest blood glucose levels were associated with relatively high lactate levels which had decreased an hour

later, in all but one the lactate levels were less than 20 mg/100 ml., similar to those observed in the well-oxygenated infant monkeys, and were associated with O_2 saturations of 54–77 % (Dawes *et al.* 1960, Table 1). Thus the degree of hypoxia, when present, was slight, possibly because these blood samples had been taken while the foetus was still *in utero*. In the foetal lambs the blood samples had been taken after delivery, though with the umbilical cord intact, and the lactate levels were rather higher than in the monkeys. Nevertheless, in all but four of the younger lambs (up to 91 days gestation age), the blood lactate was less than 30 mg/100 ml. and in only two was there more than 40 mg/100 ml. In the older lambs (111–146 days

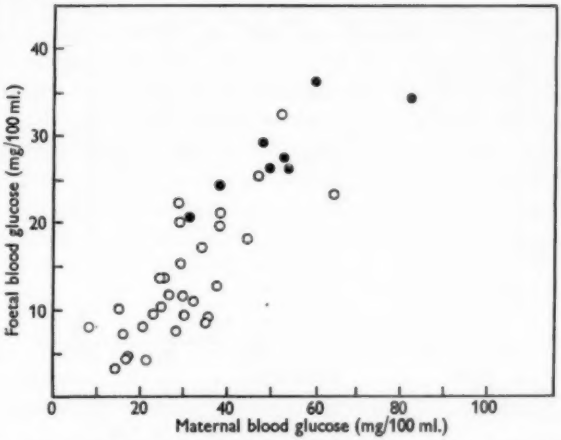


Fig. 2. Foetal blood glucose plotted against maternal blood glucose in lambs (○) and monkeys (●).

gestation age) evidence of hypoxia was present more often, and in all but three the blood lactate was more than 30 mg/100 ml. However, there was no correlation between blood lactate and glucose in the lambs, perhaps because in this species anoxia has a relatively slight effect on foetal blood glucose (Dawes *et al.* 1959). Only in two lambs (146 days gestation age) with blood lactates of 80–90 mg/100 ml. and blood glucose levels of 23–25 mg/100 ml. was there evidence of hyperglycaemia associated with a high blood lactate; a third foetus (127 days gestation age) with a blood lactate of 79 mg/100 ml. had a blood glucose of only 14 mg/100 ml. A much younger foetus (71 days gestation age) with the exceptionally high blood glucose of 32 mg/100 ml. had a blood lactate of only 27 mg/100 ml.

Since the wide scatter in foetal blood glucose levels could not be correlated with blood lactate, it was next examined in relation to maternal

blood glucose. Figure 2, in which foetal blood glucose has been plotted against the corresponding maternal level, shows that there is a close relationship, the foetal blood glucose being maintained at about half the maternal level in both species. The relatively high blood glucose levels of 23–32 mg/100 ml. observed in three lambs were associated with the three highest maternal blood glucose levels of 47–65 mg/100 ml. and were within the same range as the monkeys. Moreover, since in the sheep the maternal blood glucose nearly always fell by 4–17 mg/100 ml. during the

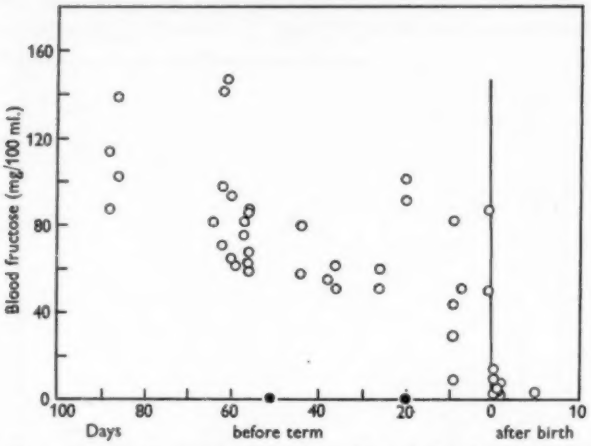


Fig. 3. Changes in blood fructose concentration with age in foetal and infant lambs (○) and monkeys (●). Conventions as for Fig. 1.

experiment, this would explain the low blood glucose levels observed in the second or third members of sets of twins or triplets and their inverse relationship to the period of anaesthesia.

Figure 1 also shows that the blood glucose in the infant lambs and monkeys was higher than in the foetuses. In five lambs the blood glucose was 48–65 mg/100 ml. within 24 hr of birth, but nine lambs less than 24 hr old had levels below 35 mg/100 ml., all but one lamb 2–7 days old had blood glucose levels of 29–49 mg/100 ml., and the older lambs had levels of 40–66 mg/100 ml. This suggests that the blood glucose was rising steadily over the first 10–15 days after birth and this was supported by observations on individual lambs. Thus in three experiments where samples were taken from the same lamb at different ages, the mean blood glucose levels on the first, third and twelfth days after birth were 21·5, 42·3 and 61·5 mg/100 ml. Although the blood glucose level in the foetal monkeys was about twice that in the foetal lambs, the levels in the infant monkeys 8–12 days old were usually lower than in lambs of the same age and were

similar to those observed in the adult pregnant monkeys (Fig. 2). Higher values, 85–90 mg/100 ml., were observed in the 15-day infant and the adolescent monkeys but these could have been due to excitement, as there was some delay between catching the monkeys and taking the samples.

Fructose. In confirmation of Hitchcock (1949), Barklay, Haas, Huggett, King & Rowley (1949) and Huggett, Warren & Warren (1951), the blood fructose level in the foetal lambs was very much higher than either the foetal or maternal blood glucose; it declined slowly with increasing gestational age and fell to very low values within a few hours of birth (○, Fig. 3). The wide range of blood fructose values at any given age could not be correlated with either foetal blood lactate or maternal blood glucose, though the lowest level recorded, 8 mg/100 ml. at 138 days gestation age, was associated with the exceptionally low maternal blood glucose of 8 mg/100 ml. Moreover, it was frequently observed that the blood fructose levels in the second and third members of sets of twins and triplets, sampled after a longer period of anaesthesia, were higher than in the first. Chinard, Danesino, Hartmann, Huggett, Paul & Reynolds (1956) have shown that there are negligible amounts of fructose in the blood of foetal rhesus monkeys; none was detected in the blood of two foetal monkeys in the present series (117 and 148 days gestation age, ●, Fig. 3).

Tissue carbohydrate

The method used to measure tissue carbohydrate (Kemp & Kits van Heijningen, 1954), gives an estimate of the total carbohydrate present, the chief constituents of which are glycogen, glucose and fructose (Mendel *et al.* 1954). The fructose content of the tissues from foetal lambs was determined separately but, as indicated earlier (Dawes *et al.* 1959), the amounts present, less than 2 mg/g, were usually negligible compared to the total carbohydrate; and since fructose was not detected in the blood of the foetal monkeys, it was assumed that the tissue fructose in this species would be even lower than in the lambs. Histological examination of the tissues suggested that their glycogen content was proportional to their total carbohydrate and it is therefore probable that the changes shown in Figs. 4–7 took place mainly in the glycogen fraction.

Liver. Figure 4 shows the changes in liver carbohydrate with age in foetal and infant lambs (○) and monkeys (●). Lambs of 58–90 days gestation age (89–57 days before term) had relatively little liver carbohydrate, less than 14 mg/g, but thereafter there was a rapid increase, levels of 60 and 98 mg/g being observed in two lambs at 138 days gestation age. The lowest values, less than 8 mg/g at 62–56 days before term, were all obtained from lambs with a low blood glucose after several hours anaesthesia; it was observed that their siblings, sampled earlier, usually had

slightly more liver carbohydrate even though they had been asphyxiated by tying the umbilical cord (Dawes *et al.* 1959). The results obtained from the foetal monkeys suggest that, as in the lambs, liver carbohydrate is high (more than 60 mg/g) by the 115th day of gestation, but since the gestation period is about 21 days longer than in the sheep the level is very much higher than in lambs at this time interval (53 days) before term. The data obtained from the infant lambs suggest that liver carbohydrate falls to less than 20 mg/g within a few hours of birth but is rising again towards the adult level by 5 days of age. The values obtained from the

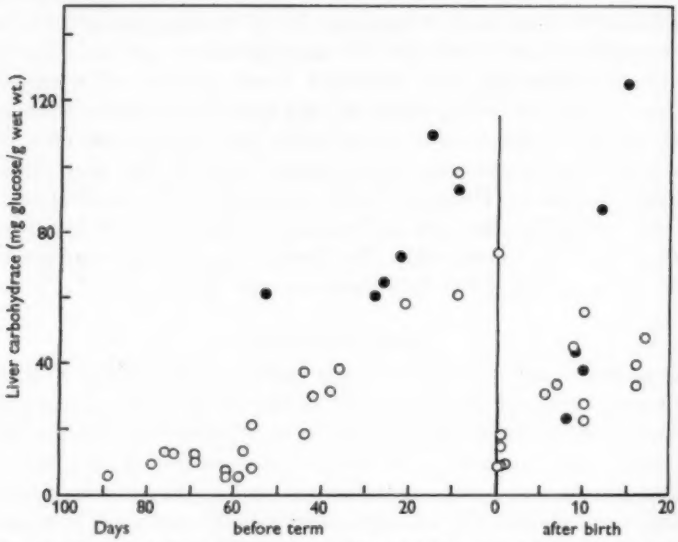


Fig. 4. Changes in liver carbohydrate with age in foetal and infant lambs (○) and monkeys (●). Conventions as for Fig. 1.

infant monkeys are probably low, since they were obtained after a 2–3 hr experiment during which a hyperglycaemia of +37–62 mg/100 ml. had occurred, but they suggest that, in this species, liver carbohydrate may rise very steeply at 8–15 days after birth and may regain the foetal level. Liver carbohydrate levels of 21 and 25 mg/g were observed in the adolescent monkeys and 34 and 46 mg/g in two of the adult pregnant monkeys; the third adult was discovered at autopsy to have a severe chronic respiratory infection, was in poor condition and had a liver carbohydrate of only 8·5 mg/g.

Data from lambs and monkeys where there was evidence of hypoxia (high blood lactates and liver lactates of more than 0·8 mg/g in lambs and

more than 0·4 mg/g in monkeys) have been excluded, since in these animals, all in the last third of gestation, the liver carbohydrate was usually low compared with the values in Fig. 4. Thus two lambs (127 and 146 days gestation age) with blood lactates of 80–90 mg/100 ml. had liver carbohydrates of only 13·0 and 16·3 mg/g, even lower than the values of 26–45 mg/g observed in lambs of this age asphyxiated by tying the umbilical cord (Dawes *et al.* 1959). Similarly three foetal monkeys (one from the adult with a respiratory infection, one where the mother had had an apnoeic episode and the one born vaginally at 117 days gestation), where there was evidence of hypoxia, had liver carbohydrates of 24–42 mg/g, whereas those asphyxiated deliberately by dividing the cord had liver carbohydrates of 41–73 mg/g; a fourth monkey (No. 110, Dawes *et al.* 1960), where the cord was divided after a long period of hypoxia, had a liver carbohydrate of only 15 mg/g. The two infant monkeys breathing 100% nitrogen had terminal liver carbohydrate levels of 37 and 53 mg/g, similar to values observed in unasphyxiated infants of the same age.

Liver fructose fell slightly with increasing age; it was 1·6 mg/g in two lambs of 89 and 91 days gestation age and 0·5–1·3 mg/g in four lambs of 126–138 days gestation age.

Lungs. Dawes *et al.* (1959) found that the lung carbohydrate in lambs asphyxiated by tying the umbilical cord was of the same order as that in their unasphyxiated siblings, and a similar conclusion was reached in the work on foetal and infant monkeys (Dawes *et al.* 1960). Although the values shown in Fig. 5 for foetal lambs were all obtained from unasphyxiated animals, the number of unasphyxiated monkeys available was small and Fig. 5 shows results obtained on both asphyxiated and unasphyxiated monkeys. In the foetal lambs (○), lung carbohydrate rose from less than 15 mg/g at 58–71 days gestation age (89–76 days before term) to peak values of 16–27 mg/g at 85–109 days gestation age, after which it declined rapidly to less than 6 mg/g near term. High values, 14–19 mg/g, were observed in the foetal monkeys (●) at 115–117 days gestation age and there was a rapid decline to less than 8 mg/g at approximately the same time interval before term, as in the lambs, though at a slightly older gestational age. The lung carbohydrate concentration in the 5–17-day-old lambs and the infant monkeys was even lower, less than 4 mg/g, and values of 0·7–1·7 mg/g were observed in the adolescent and adult monkeys.

In the lambs lung fructose fell from 1–2 mg/g at 61–91 days gestation age to 0·5–1·1 mg/g at 126–138 days gestation age.

Heart. Figure 6 shows that the lambs (○) of less than 100 days gestation age (more than 47 days before term) had cardiac carbohydrates of 20–40 mg/g with a peak value at about 91 days gestation age; after this there was a decline to 14–22 mg/g nearer term, 11–21 mg/g in the new-born

lambs and 6–11 mg/g in the older lambs. A similar decline was observed in the monkeys (●) where the level was 23·5 mg/g at 115 days gestation age (53 days before term), 10–19 mg/g nearer term and 4–5 mg/g in the infants and adults. Since it is known that cardiac glycogen is very rapidly depleted in anoxia (Dawes *et al.* 1959, 1960), care has been taken to exclude from Fig. 6 all data where there was evidence of hypoxia. The lambs included in Fig. 6 had blood lactates of less than 42 mg/100 ml. (in all but two they were less than 30 mg/100 ml.) and all but one monkey had blood lactates of less than 20 mg/100 ml. The cardiac lactate concentration was

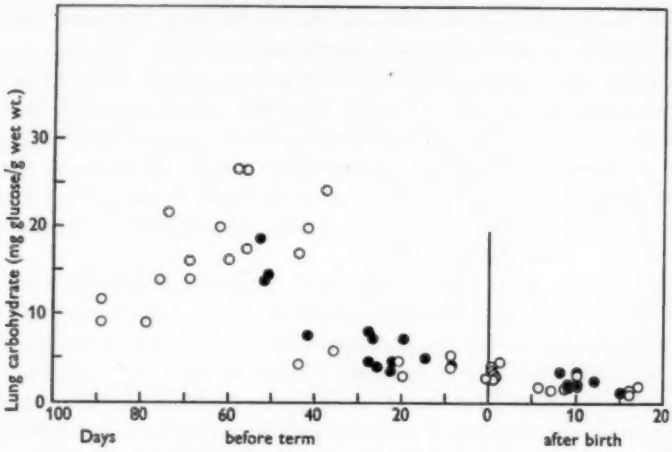


Fig. 5. Changes in lung carbohydrate with age in foetal and infant lambs (○) and monkeys (●). Conventions as for Fig. 1.

0·4–1·6 mg/g in the lambs (less than 1·0 mg/g in all but two) and 0·7–1·3 mg/g in the monkeys.

Cardiac fructose was 1·1–1·2 mg/g in the younger lambs and 0·3–0·8 mg/g in lambs nearer term (126–138 days gestation age).

Skeletal muscle. Like lung carbohydrate, skeletal muscle carbohydrate is relatively unaffected by acute asphyxia in either lambs or monkeys (Dawes *et al.* 1959, 1960) and therefore Fig. 7, like Fig. 5, includes results from both asphyxiated and unasphyxiated monkeys, though those shown for lambs (○) were all obtained from unasphyxiated animals. In lambs of less than 90 days gestation age (more than 57 days before term) the muscle carbohydrate was less than 14 mg/g, but thereafter it increased rapidly and lambs of more than 100 days gestation age had muscle carbohydrates of 20–47 mg/g. Similarly, the monkeys (●) had muscle carbohydrates below 20 mg/g at 51–53 days before term, but nearer term the level in-

creased to 19–34 mg/g; thus the increase in muscle carbohydrate occurred at approximately the same time interval before term as in the lambs, but at a slightly older gestational age. The wide scatter in the older lambs existed not only among individuals from different ewes but also between litter-mates. In general, comparing lambs or monkeys of the same age, those with a high muscle carbohydrate tended to have higher carbohydrate levels in the other tissues, though there was no correlation with blood glucose concentration. It is noteworthy that the four lambs (gestation

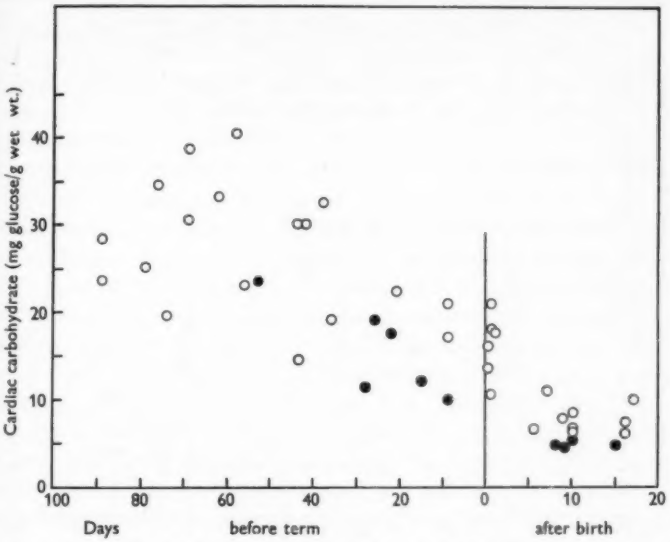


Fig. 6. Changes in cardiac carbohydrate with age in foetal and infant lambs (○) and monkeys (●). Conventions as for Fig. 1.

age 140 days) from the ewe with pregnancy toxaemia had muscle carbohydrates within the normal range.

Figure 7 also illustrates the dramatic fall in muscle carbohydrate which occurred in the lambs after birth. In lambs 3–12 hr old it had already fallen to 11–27 mg/g and lambs more than 24 hr old had muscle carbohydrates of only 7–15 mg/g, similar to values obtained by the same method in adult sheep (Shelley, unpublished). In the three experiments where muscle biopsy samples were taken from the same lamb at different ages, the mean values observed on the first, third, seventh and twelfth days after birth were 19·6, 9·7, 11·7 and 10·9 mg/g and one of these had a muscle carbohydrate of only 7·1 mg/g 41 days after birth. Whereas one lamb from a set of triplets had a muscle carbohydrate of 26·8 mg/g 5 hr after birth, its

litter-mates had less than 8 mg/g on the third and fourth days after birth. The extremely low value of 2·3 mg/g was obtained from one 9-day-old lamb and is of interest because this lamb was in poor physical condition with signs of muscular weakness. With one exception the 8–15-day-old monkeys had muscle carbohydrates within the same range as lambs of this age, slightly higher than the values of 5–9 mg/g observed in the adolescent and adult monkeys. The one exception (No. 101, Dawes *et al.* 1960), where the level was 18·6 mg/g 12 days after birth, is noteworthy in that this monkey had been born prematurely at 150 days gestation age and, though

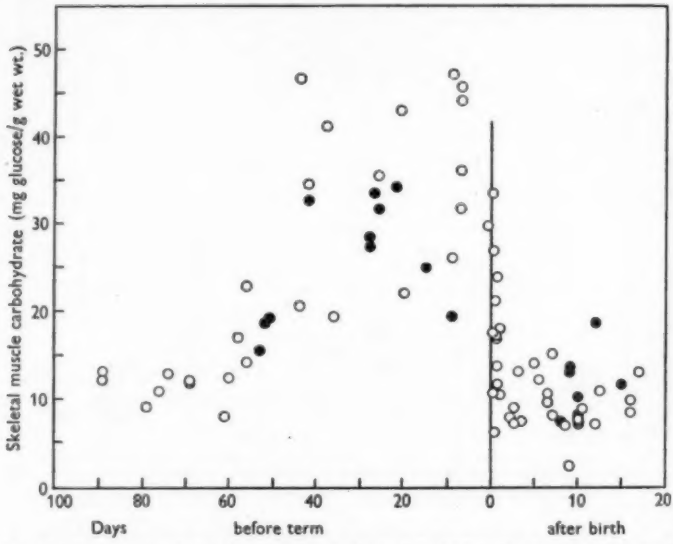


Fig. 7. Changes in skeletal muscle carbohydrate with age in foetal and infant lambs (○) and monkeys (●). Conventions as for Fig. 1.

in good condition, was smaller and possibly less active than the other infants. Thus in the monkeys, as in the lambs, muscle carbohydrate probably fell after birth.

Skeletal muscle fructose was 1·1–1·5 mg/g in the younger foetal lambs, 0·3–0·9 mg/g in lambs of 126–138 days gestation age and less than 0·3 mg/g in two lambs 3 and 10 hr after birth.

Other tissues. There was no change in either brain or kidney carbohydrate with increasing age in the foetal lambs. Thus the range for brain carbohydrate was 0·5–1·4 mg/g in lambs of 83–91 days gestation age and 0·5–1·6 mg/g at 111–146 days gestation age; the corresponding values for brain fructose were 0·4–0·6 mg/g and 0·3–0·6 mg/g in the two groups.

Slightly higher values were obtained for kidney carbohydrate, 2·8–4·6 mg/g in the younger lambs and 3·5–5·8 mg/g in the older lambs; the corresponding fructose concentrations were 0·9–1·7 mg/g and 0·6–2·0 mg/g.

Placental carbohydrate in the rhesus monkey probably falls in the last third of pregnancy, since there was 8·2 mg/g in the placenta from the one unasphyxiated foetus at 115 days gestation age and only 0·6–2·6 mg/g at 140–158 days gestation age. In one ewe at 144 days gestation age, where tissue samples were taken from various parts of the placenta, there was a mean carbohydrate concentration of 3 mg/g with 4·2 mg/g in the endometrium (H. B. Parry and H. J. Shelley, unpublished).

Total body carbohydrate. The total carbohydrate content of the liver, lungs, heart, skeletal muscle, brain and kidneys and the glucose content of the blood were calculated for each of nine foetal lambs from either known or calculated organ weights, taking the blood volume as 11–15% of body weight depending on gestational age (Carlyle, 1948; Wallace, 1948). The sum of these values gave an approximate figure for total body carbohydrate, since it is probable that these tissues contain the greater part of the carbohydrate present. In practice, since skeletal muscle accounted for 22–52% of the body weight as compared to 2–9% for the liver and even smaller percentages for the other organs, it was found that skeletal muscle carbohydrate accounted for 67–92% of the total body carbohydrate, liver carbohydrate for up to 30% and the other tissues for only 2–3%, the contribution of the brain and kidneys being negligible, less than 1%. Comparable values for total body carbohydrate were therefore obtained for the other foetal lambs, the infant lambs, and the foetal and infant monkeys where brain and kidney carbohydrate had not been measured; in the monkeys total skeletal muscle mass was taken as 30% of body weight and blood volume as 10% for foetal monkeys and 6% for infant monkeys (Gregersen, Sear, Rawson, Shu Chien & Saiger, 1959). However, it must be emphasized that because these calculations assume that the carbohydrate was distributed evenly in each tissue, the values obtained are only approximations. Since skeletal muscle carbohydrate formed such a large percentage of the total, its distribution was investigated in two foetal lambs (127 days gestation age) in each of which samples from 11 different muscles were analysed; the variation from muscle to muscle was considerable, 14–40 mg/g in one lamb, 21–42 mg/g in the other, but in both lambs the mean value was more than 30 mg/g and was similar to the concentration in the semitendinosus, the muscle usually taken for analysis. Muscle carbohydrate distribution was also investigated in fourteen infant lambs 2½ hr–17 days old, in each of which samples from at least five muscles were analysed; again, the concentration in the semitendinosus did not differ greatly from the mean.

These calculations revealed several points of interest. First, with the increase in weight of the foetal lambs from less than 100 g at 58 days gestation age to 549–1108 g at 85–91 days gestation age there was a proportional increase in total body carbohydrate from less than 0·5 g/foetus to 3–6 g/foetus. Thereafter, however, mainly owing to the large increase in liver and skeletal-muscle carbohydrate, the total body carbohydrate increased out of all proportion to body weight, to 29–38 g/foetus in lambs weighing 2·3–4·4 kg at 126–146 days gestation age. Calculated in terms of body weight, the total carbohydrate content of the foetal lambs increased from 5·7–8·8 g/kg (mean 7·5 g/kg) at 58–91 days gestation age to 7·1–14·1 g/kg (mean 10·6 g/kg) at 103–146 days gestation age.

Secondly, considering those tissues where there was a decrease in carbohydrate concentration in the last third of gestation, whereas in the lungs there was a decrease in the total amount present, from 559–1420 mg in lambs of 85–109 days gestation age to 162–530 mg at 111–146 days gestation age, in the heart the increase in weight more than made up for the decrease in cardiac carbohydrate concentration and the total cardiac carbohydrate increased from 133–161 mg in lambs of 85–91 days gestation age to 305–395 mg at 103–146 days gestation age.

Thirdly, although the foetal monkeys were only a tenth of the weight of the mature foetal lambs and their absolute carbohydrate content was therefore very much smaller, when expressed per kilogram body weight their total body carbohydrate was almost identical with that of the lambs. Thus the level was 7·7 g/kg in the unasphyxiated monkey at 115 days gestation age but had increased to 9·0–12·6 g/kg (mean 10·5 g/kg) at 140–158 days gestation age. As in the lambs, the total lung carbohydrate decreased with increasing gestational age but, in contrast, there was no change in total cardiac carbohydrate.

Finally, the changes occurring after birth were of particular interest. Table 1 gives the mean absolute values for skeletal muscle, liver and total body carbohydrate in the mature foetal and the younger infant lambs and monkeys and, by subtraction, the mean differences in tissue carbohydrate between the foetuses and infants of each species. The infants of both species were directly comparable with the mature foetuses since little gain in weight had occurred within the first 24 hr of birth in the lambs or 8–10 days in the monkeys (Dawes *et al.* 1960). The results from one 2½-hr-old lamb, born prematurely at 142 days gestation age, have been omitted, since in this lamb the liver and muscle carbohydrate were still within the foetal range (Figs. 4 and 7). Since the skeletal muscle carbohydrate represented 80% of the total carbohydrate in the mature foetal lambs and 76% of that in the foetal monkeys, and since the amount present in the infants was only 48–58% of that in the foetuses, the decrease

in muscle carbohydrate after birth represented a loss of 42% of the total foetal carbohydrate in the lambs and 32% in the monkeys and was considerably greater than the total carbohydrate content of the liver, 2·4 times the foetal liver carbohydrate in the lambs and 1·5 times that in the monkeys. This suggests that the foetal skeletal muscle carbohydrate in these two species constitutes a carbohydrate store about half of which is used up shortly after birth, and that quantitatively it is probably more important than liver glycogen. Table 1 also emphasizes even further the similarity between the lambs and monkeys, since the decrease in total

TABLE 1. Total body carbohydrate in mature foetal and infant lambs and rhesus monkeys

		Tissue carbohydrate (g/foetus or infant)			Total carbohydrate	
	No. of animals	Muscle	Liver	Other tissues	(g/foetus or infant)	(g/kg body wt.)
Lambs:						
Foetus, 126–146 days gestation age	5	28·7*	6·4	0·8	35·9	10·3
Infant, 3–24 hr after birth	5	13·6†	1·1	0·7	15·4	4·1
Difference		15·1	5·3	0·1	20·5	6·2
Monkeys:						
Foetus, 140–158 days gestation age	6	3·1	0·9	0·1	4·1	10·5
Infant, 8–10 days after birth	5	1·8	0·6	0·1	2·5	4·4
Difference		1·3	0·3	0	1·6	6·1

The mean total body carbohydrate has been calculated in two ways: (1) in g/foetus or infant as the sum of the mean values for each tissue, and (2) in g/kg body wt. from the total body carbohydrate for each animal. * Mean of 9 observations; † mean of 11 observations.

carbohydrate in g/kg body weight and the final levels reached in the infants were almost identical in the two species; the final levels (4·1 and 4·4 g/kg) were considerably lower than any observed in the foetuses.

However, the lambs and monkeys did differ considerably in their rates of growth after birth. Whereas the lambs had almost doubled their birth weight within 8–10 days of birth and had quadrupled it by the middle of the third week, the monkeys were only about 25% heavier by 8–10 days of age (Dawes *et al.* 1960) and would have been only 30–40% heavier by the middle of the third week (H. N. Jacobson, personal communication). Since in both species the various organ weights increased in roughly the same proportion as total body weight, it can be seen that in the lambs, although the carbohydrate concentration in the skeletal muscle and liver did not regain the foetal level, the total amount laid down very soon exceeded that present in the mature foetus. It is possible that the much

steeper rise in liver carbohydrate concentration and its restoration to the foetal level in the infant monkeys (●, Fig. 4) was associated with the much slower rate of growth in this species as compared with the lambs.

DISCUSSION

Foetal tissue glycogen in different species

Although it is just over one hundred years since Claude Bernard (1859*a*) identified glycogen in foetal tissues and described qualitatively the changes in content occurring in the different tissues during gestation, there have been no attempts to make as detailed a quantitative survey. Nevertheless, sufficient information is available to suggest that changes in tissue glycogen similar to those observed in the foetal lambs and monkeys also occur in other species, though their time relationships with respect to parturition may vary from species to species.

Liver. Bernard was unable to detect glycogen in the livers of foetal lambs during the first half of gestation, but with improved methods of extraction Pflüger (1903) showed that a small amount, 7·9 mg/g, was present in the liver of a 2-month-old foetal lamb, an estimate similar to those for total carbohydrate obtained in lambs of this age in the present work. In the older lambs, from 100 days gestation age onwards, i.e. in the last third of gestation, the liver carbohydrate increased steadily (Fig. 4) until near term it was considerably higher than the normal adult level of 40 mg/g (Parry & Shelley, 1958). A similar trend was observed by Aron (1922), but his values are much lower than those reported here, possibly because he estimated glycogen, not total carbohydrate, and had partially asphyxiated the foetuses by killing their mothers before delivery, a circumstance which would be expected to deplete liver glycogen near term (Dawes *et al.* 1959); one advantage of the present method of carbohydrate analysis is that the products of glycogenolysis as far as glucose 1-phosphate and free glucose are all included in the assay (Mendel *et al.* 1954). Liver glycogen levels of up to 90 mg/g have been observed in new-born lambs (Roderick, Harshfield & Hawn, 1937), in good agreement with the present results on mature foetuses. In the foetal monkeys liver carbohydrate was present in large amounts at an earlier stage of gestation than in the lambs and near term the concentration was 92–109 mg/g (Fig. 4), again much higher than in the adults. In man foetal liver glycogen starts to rise during the second third of gestation (Szendi, 1936; Villee, 1953*a*, 1954) and values of 40–50 mg/g were observed at 105–140 days gestation age (Villee, 1954); reliable information on older human foetuses is lacking, but Szendi's results suggest that the liver glycogen increases steadily up to term.

Similar rises in liver glycogen in the latter part of gestation have been observed in other species, though it is possible that some of the values given for foetuses near term are low, since the mother was usually stunned or killed before delivering the foetuses. In another ungulate, the pig, foetal liver glycogen also begins to rise at 105–120 days gestation age (Mendel & Leavenworth, 1907; Aron, 1922), though since the gestation period is only 120 days, the rise occurs nearer term than in the lambs; the livers of new-born piglets contain 85 mg/g (McCance & Widdowson, 1959). Liver glycogen also rises late in gestation in rodents; in rats the rise occurs in the last fifth of gestation to a maximum of 106 mg/g at term (Corey, 1935*a*; Stuart & Higgins, 1935; Martínek & Mikuláš, 1954; Jacquot, 1955), in rabbits in the last quarter to about 40 mg/g at term (Lochhead & Cramer, 1908; Snyder & Hoskins, 1928; Szendi, 1936; Jost & Jacquot, 1955) and in guinea-pigs during the last fifth to about 30 mg/g at term (Aron, 1922; Hard, Reynolds & Winbury, 1944). Foetal liver glycogen is still rising in the last fifth of gestation in dogs (Schlossmann, 1938) and this work is of particular interest in that the foetuses were delivered under general anaesthesia as in the present work and values for liver total carbohydrate of up to 117 mg/g were obtained near term, similar to those in the lambs and monkeys and to the liver glycogen of new-born puppies (Demant, 1887); values of up to 97 mg/g were also observed in mature foetal guinea-pigs delivered under general anaesthesia (Shelley, unpublished), in contrast to the much lower values obtained by earlier workers (see above). Schlossmann also showed that the total carbohydrate content of the foetal livers exceeded their glycogen content by less than 1%, suggesting that the results obtained in the present work give a reliable estimate of liver glycogen.

Lungs. Lung carbohydrate is not depleted by acute asphyxia in foetal lambs (Dawes *et al.* 1959) and this may explain why previous observations on foetal lung glycogen are in better agreement with the present results than were those on liver glycogen. Calculation from the results of Fauré-Fremiet & Dragoiu (1923) on foetal lambs show that, as in the present work, peak values of up to 28·5 mg/g were observed just over half way through gestation, after which the level dropped abruptly. They confirmed Bernard's observations that the glycogen was concentrated in epithelial tissue and estimated that this must contain 75–78 mg/g; the fall in glycogen concentration was associated with structural alterations in the lungs during which the epithelial tissue disappeared. In man, also, lung glycogen is concentrated in the alveolar epithelium and reaches a peak value about half way through gestation (Szendi, 1936); the values of 27 mg/g at 19 weeks and 3 mg/g near term observed by Villee (1953*a*, 1954) are in excellent agreement with the peak and full-term values in the lambs (Fig. 5). Monkeys half way through gestation were not available but Fig. 5 shows

that lung carbohydrate was falling in the last third of gestation and, again, the fall was associated with the disappearance of the alveolar epithelium. In rabbits the peak lung glycogen concentration occurs rather late, in the last fifth of gestation (Szendi, 1936), but, as in the other species, it decreases to 3–4 mg/g near term.

Heart. Whereas all the species so far investigated have high liver glycogen and low lung glycogen concentrations at term, regardless of their relative maturity, cardiac glycogen appears to vary inversely with maturity. Thus in lambs and monkeys, which are able to stand or crawl within an hour of birth and are born covered with wool or fur and with their eyes open, the cardiac carbohydrate fell during the last third of gestation to 10–20 mg/g near term (Fig. 6): values of 30–40 mg/g had been observed in lambs just over half way through gestation, comparable to the cardiac glycogen in human foetuses in the first half of gestation (Villee, 1954). There are no quantitative estimates of cardiac carbohydrate in foetuses of other species, but new-born rats, which are relatively immature at birth, have 22–31 mg/g, new-born rabbits have 5–13 mg/g, and the exceptionally mature new-born guinea-pig has only 4–5 mg/g (Dawes *et al.* 1959), similar to the level in the infant and adult monkeys and in other adult mammals (Cruickshank, 1936); new-born piglets have 15 mg/g (McCance & Widdowson, 1959) and new-born kittens have 11–14 mg/g (Shelley, unpublished).

Skeletal muscle. Foetal skeletal muscle carbohydrate also appears to be related to maturity and it is tempting to argue that species which are capable of vigorous muscular activity soon after birth need larger amounts of muscle glycogen than those born in a less mature condition. Thus in the lambs and monkeys the muscle carbohydrate increased during the last third of gestation from less than 20 mg/g to 20–47 mg/g near term (Fig. 7). Similarly, carcass glycogen in foetal piglets increases to 11 mg/g near term (Mendel & Leavenworth, 1907), a value in excellent agreement with the calculated whole-body carbohydrate in the full-term lambs and monkeys; muscle from new-born piglets may contain more than 70 mg/g (McCance & Widdowson, 1959). Foetal guinea-pigs have a muscle carbohydrate concentration of 19–25 mg/g near term, but the less mature full-term foetal rabbit has only 13–17 mg/g (Shelley, unpublished) and the full-term foetal rat has a muscle glycogen of only 12 mg/g (Martínek & Mikuláš, 1954); foetal puppies near term have 14–21 mg/g (Schlossmann, 1938). Nevertheless, in all these species the foetal muscle carbohydrate concentration near term was at least twice the corresponding adult level.

Other tissues. The suggested decline in placental glycogen during the latter part of gestation in the monkeys would be in agreement with observations on man (Szendi, 1934), rats (Corey, 1935*a*) and rabbits (Lochhead & Cramer, 1908; Huggett, 1929; Loveland, Maurer & Snyder,

1931; Szendi, 1934) where the level begins to fall at about the time that foetal liver glycogen begins to rise. In sheep and cows the placenta is relatively poor in glycogen throughout gestation but glycogen-rich 'plaques' on the inner surface of the amnion and the umbilical cord disappear in the latter part of pregnancy, again at the stage at which foetal liver glycogen accumulates (Bernard, 1859*b*). In the foetal lambs' kidneys, the glycogen was concentrated in the tubular epithelium and, as in man (Villee, 1953*a*), remained relatively constant throughout the last two-thirds of gestation. Although in the lambs the carbohydrate content of the cerebral cortex did not appear to change with increasing gestational age, the amounts present were so small as to be near the limit of the method; Villee (1954) has observed a decrease in the glycogen content of human foetal brains in the first half of gestation.

Blood sugars. In the lambs and monkeys the foetal blood glucose was closely related to the maternal level (Fig. 2) and did not change with increasing gestational age (Fig. 1), but in other species, although the foetal level was always lower than the maternal level, a rise in foetal blood glucose was usually observed towards the end of gestation (Aron, 1923; Snyder & Hoskins, 1928; Britton, 1930; Schlossmann, 1931, 1938; Corey, 1932; Hard *et al.* 1944). It is possible that this rise was associated with accidental asphyxia at a time when large amounts of glycogen were present in the liver, a factor which was excluded as far as possible in the present work. Values of about 30 mg/100 ml., similar to those in the foetal monkeys, were observed in several species earlier in gestation, and it is probable that the higher level in these species, as compared with lambs, is due to the higher level in the adults as compared with adult sheep, rather than to differences in foetal metabolism. The similarity in the ratio of foetal to maternal blood glucose in the lambs and monkeys was surprising, because whereas the sheep placenta converts part of the foetal blood glucose into fructose (Huggett *et al.* 1951; Alexander, Andrews, Huggett, Nixon & Widdas, 1955), the monkey placenta at this stage of gestation does not (Chinard *et al.* 1956). The fall in blood fructose with increasing gestational age in the lambs (Fig. 3) was reflected in the tissues, which near term usually contained less than 1 mg/g.

Factors affecting foetal tissue glycogen

Little is known about the factors responsible for the appearance of glycogen in foetal tissues, but the present work makes it clear that exogenous factors alone cannot be responsible. Although there is a correlation between the appearance of islets of Langerhans in the foetal pancreas and the increase in foetal liver glycogen (Aron, 1922, 1931) and although the latter is dependent on foetal pituitary and adrenal function (Jacquot,

1955; Jost & Jacquot, 1955), the appearance of insulin and adrenocorticoids in the foetal circulation at this late stage in gestation would not explain the much earlier deposition of glycogen in the heart, lungs and skeletal muscle. It is more likely that the appearance of glycogen in a given tissue coincides with the development in that tissue of the enzymes necessary for its synthesis, of which the 'branching enzyme' needed for the formation of glycogen 'primer' may be among the last to appear (Nemeth, Insull & Flexner, 1954). Similarly, changes in enzyme activity may well be responsible for the decrease in lung and cardiac carbohydrate concentration in the latter part of gestation and for the difference between foetal and adult skeletal muscle.

Although differences in enzyme activity could account for changes in the general pattern of foetal tissue glycogen distribution, they are unlikely to account for the variations observed between different individuals of the same age. Such variation is more likely to be associated with differences in the availability of substrates for glycogen synthesis, particularly in those tissues where glycogen is not depleted by asphyxia. The injection of insulin into pregnant rats produced foetal hypoglycaemia and a fall in foetal liver glycogen (Corey, 1932, 1935*b*) and starving pregnant rats for 22 hr (Stuart & Higgins, 1935) or treating pregnant rabbits with phlorrhizin (Lochhead & Cramer, 1908) also depleted foetal liver glycogen. Conversely, treating pregnant rats with glucose increased the foetal liver and muscle glycogen (Corey, 1935*b*), though maintaining pregnant rabbits on a supposedly carbohydrate-rich diet (carrots and cabbage) had little effect (Lochhead & Cramer, 1908). Some long-term experiments on the effects of maternal nutrition on foetal tissue glycogen are clearly needed.

The importance of foetal tissue carbohydrate

Importance to the foetus. Even less is known about the importance to the foetus of its glycogen reserves since, although observations have been made on the glycogen content of various tissues, few physiological experiments have been made. It has been assumed that foetal liver glycogen behaves in the same way as adult liver glycogen and this hypothesis is supported by the fall in liver glycogen and rise in blood glucose observed in foetal lambs and monkeys asphyxiated by tying the umbilical cord (Dawes *et al.* 1959; 1960) and by its depletion in the presence of foetal hypoglycaemia (see above). Thus foetal liver glycogen is highly labile and Goldwater & Stetten (1947) estimated its rate of turnover in the foetal rat to be 5 mg/g/hr. Since large amounts of glycogen are present in the placenta and lungs at a time when very little glycogen is present in the liver, it has been suggested that these act as temporary glycogen stores until the liver is able to take over (Bernard, 1859*b*; Lochhead & Cramer, 1908; Szendi, 1934,

1936; Corey, 1935*a*; Villee, 1953*b*), but there is no evidence for its liberation from these organs under conditions of foetal stress. The placental glycogen of the rabbit is unaffected by adrenaline or hypoglycaemia (Huggett, 1929) and there is no change in lung carbohydrate in acutely asphyxiated foetal lambs and monkeys (Dawes *et al.* 1959, 1960). In contrast, there is a well-established correlation between cardiac carbohydrate concentration and the ability to survive anoxia (Dawes *et al.* 1959, 1960; Stafford & Weatherall, 1960), and since the danger of hypoxia is probably one of the chief hazards of intra-uterine life, the possession of a high cardiac glycogen concentration may be highly beneficial to the foetus and should help it to survive the trauma of birth.

Importance to the new-born animal. The changes in tissue glycogen in new-born animals suggest that both liver glycogen and skeletal muscle glycogen are important stores for use immediately after birth. A profound fall in liver glycogen occurs within a few hours of birth in dogs (Demant, 1887), guinea-pigs (Hard *et al.* 1944), rats (Martínek & Mikuláš, 1954; Stafford & Weatherall, personal communication), lambs and possibly monkeys (Fig. 4) and may be responsible for the initial rise in blood glucose observed in the lambs (Fig. 1) and guinea-pigs (Hard *et al.* 1944) soon after birth. The liver glycogen remained low, below 20 mg/g, for about a fortnight after birth in the dogs, rats and guinea-pigs but was rising again within a week of birth in the lambs and monkeys. The blood glucose level also rises in human babies after birth (Desmond, Hild & Gast, 1950; Cornblath, Levin & Marquetti, 1958) and liver glycogen also falls in chickens after hatching (Needham, 1942). A fall in skeletal muscle carbohydrate after birth has been observed not only in kittens (Bernard, 1859*a*) and in lambs and monkeys (Fig. 7), but also in the much less active new-born rat (Martínek & Mikuláš, 1954). In the last three species the level fell to less than half that in the full-term foetus; whereas in the lambs this occurred within 24 hr of birth, the fall was slower in the rat and, since the initial value was low (12 mg/g), the decrease in muscle glycogen was less. The rate of fall in the infant monkey is not known, since all those available were at least 8 days old, but in the kitten the fall occurs in the first 24 hr after birth. The rough calculations of total body carbohydrate in Table 1 suggest that in the lambs and monkeys the total fall in muscle carbohydrate was several times greater than the fall in liver carbohydrate, and similar calculations suggest that even in the rat the amount of glycogen lost from the muscles was at least half that disappearing from the liver. Whereas in all the species studied there was a rise in liver glycogen 10–16 days after birth, there was no such rise in muscle glycogen.

These observations suggest that the metabolic requirements of new-

born animals are so great that their carbohydrate reserves are utilized in the first few hours after birth, and that they are unable to obtain sufficient food both to satisfy these needs and to restore their liver glycogen until more than a week later, and can never restore their muscle glycogen. It is known that in foetal rats near term the turnover rates of several substances, including glycogen, are several times greater than in the adult (Goldwater & Stetten, 1947) and there is no reason to suppose that they are any lower in the new-born animal; indeed, the increase in oxygen consumption observed after birth in many species, including lambs, monkeys and rats, suggests that the turnover rates may actually increase in new-born animals (Gelineo, 1954; Dawes & Mott, 1959; Mount, 1959; Dawes *et al.* 1960; Taylor, 1960). The drastic effects of starving new-born animals are particularly well illustrated in the pig, where in the absence of food the rise in blood glucose occurring after birth is soon succeeded by hypoglycaemia and death (Goodwin, 1957). In some species, notably man, lactation is not fully established until 24–48 hr after parturition and during this period the new-born animal must be largely dependent on its own reserves, of which the liver and muscle carbohydrate evidently form an important part; for instance, McCance & Widdowson (1959) estimated that carbohydrate accounted for 84% of the solids metabolized by starved new-born piglets, 67–69% being contributed by muscle and liver glycogen. It is therefore possible that factors which reduce the glycogen content of the foetus *in utero* may well jeopardize the infant's chances of survival after birth. Although acute anoxia only partially depletes liver carbohydrate and does not affect skeletal muscle carbohydrate in foetal lambs and monkeys (Dawes *et al.* 1959, 1960), the results obtained on one particular monkey (No. 110, 145 days gestation age, Dawes *et al.* 1960), where placental infarcts were present and there was evidence of prolonged hypoxia, suggest that a prolonged period of placental insufficiency may not only deplete liver carbohydrate to a greater extent than does acute anoxia, but may also affect muscle carbohydrate; the latter was only 15 mg/g in this foetus, less than half the normal value. Such an infant would probably have a poor chance of survival after birth, and further work is needed to determine to what extent the deleterious effects of maternal malnutrition, placental insufficiency and birth asphyxia may be due to the presence of inadequate carbohydrate reserves in the new-born animal.

SUMMARY

1. Blood glucose and fructose and the total carbohydrate concentration in several tissues have been measured in foetal lambs in the last two-thirds of gestation. Similar observations were also made on lambs from $2\frac{1}{2}$ hr to 17 days after birth and monkeys 8–15 days after birth.

2. The blood glucose did not change with increasing gestational age and was equal to about half the maternal level in both foetal lambs and monkeys. After birth it rose rapidly to near-adult levels.

3. In the lambs blood fructose fell with increasing gestational age and reached very low levels soon after birth; none was detected in the blood of two foetal monkeys.

4. Liver carbohydrate rose during the last third of gestation in the foetal lambs, from less than 14 mg/g to 60–98 mg/g near term; monkeys in the last third of gestation had 60–104 mg/g. Observations on infant lambs and monkeys suggested that it fell soon after birth and rose again 5–10 days later.

5. In the lambs lung carbohydrate rose to up to 27 mg/g just over half way through gestation, after which it fell rapidly to 3–4 mg/g near term; a similar fall occurred in the foetal monkeys and even lower levels were observed in the infant animals.

6. The cardiac carbohydrate rose to up to 40 mg/g in lambs just over half way through gestation, after which it fell in both species; the fall continued after birth.

7. Skeletal muscle carbohydrate rose during the last third of gestation in both species to a maximum of 47 mg/g in lambs and 34 mg/g in monkeys. In the lambs it fell to less than half the foetal level within 24 hr of birth; similar low levels were observed in the infant monkeys.

8. The fructose concentration in these tissues was usually negligible compared with the total carbohydrate, less than 2 mg/g in the foetal lambs.

9. Histological examination of the tissues suggested that their glycogen content was proportional to the total carbohydrate concentration.

10. It was concluded that the results were consistent with observations on other species and that these carbohydrate stores may be of great importance, particularly to the new-born animal.

This work was carried out with the aid of grants from the Nuffield Foundation and the United Cerebral Palsy Research and Educational Foundation. I should like to thank Dr G. S. Dawes and Mr H. B. Parry for providing me with almost all the blood and tissue samples and for their interest and encouragement throughout this work, the National Institute for Neurological Diseases and Blindness and, in particular, Dr W. F. Windle for their generous hospitality in San Juan, Puerto Rico, and the staff of both the Nuffield Institute for Medical Research and the Field Station of Perinatal Physiology for their expert technical assistance.

REFERENCES

ALEXANDER, D. P., ANDREWS, R. D., HUGGETT, A. ST G., NIXON, D. A. & WIDDAS, W. F. (1955). The placental transfer of sugars in sheep: studies with radioactive sugar. *J. Physiol.* **129**, 352–366.

ARON, M. (1922). Le glycogène du foie embryonnaire. Déterminisme de sa formation. *Bull. Soc. Chim. biol., Paris*, **4**, 209–222.

ARON, M. (1923). La glycémie chez l'embryon. *C.R. Soc. Biol., Paris*, **89**, 187–189.

ARON, M. (1931). Recherches histophysiologiques sur le fonctionnement et les corrélations des glandes endocrines embryonnaires chez les vertébrés. *Bull. biol.* **65**. 438–521.

BACON, J. S. D. & BELL, D. J. (1948). Fructose and glucose in the blood of the foetal sheep. *Biochem. J.* **42**, 397–405.

BARKER, S. B. & SUMMERSON, W. H. (1941). The colorimetric determination of lactic acid in biological material. *J. biol. Chem.* **138**, 535–554.

BARKLAY, H., HAAS, P., HUGGETT, A. ST G., KING, G. & ROWLEY, D. (1949). The sugar of the foetal blood, the amniotic and allantoic fluids. *J. Physiol.* **109**, 98–102.

BERNARD, C. (1859*a*). De la matière glycogène considerée comme condition de développement de certains tissues chez le foetus avant l'apparition de la fonction glycogenique du foie. *C.R. Acad. Sci., Paris*, **48**, 673–684.

BERNARD, C. (1859*b*). Sur une nouvelle fonction du placenta. *C.R. Acad. Sci., Paris*, **48**, 77–86.

BRITTON, S. W. (1930). Maternal and fetal blood sugar changes under various experimental conditions. *Amer. J. Physiol.* **95**, 178–185.

CARLYLE, A. (1948). An integration of the total oxygen consumption of the sheep foetus from that of the tissues. *J. Physiol.* **107**, 355–364.

CHINARD, F. P., DANESINO, V., HARTMANN, W. L., HUGGETT, A. ST G., PAUL, W. & REYNOLDS, S. R. M. (1956). The transmission of hexoses across the placenta in the human and rhesus monkey (*Macaca mulatta*). *J. Physiol.* **132**, 289–303.

COREY, E. L. (1932). Placental permeability to insulin in the albino rat. *Physiol. Zool.* **5**, 36–48.

COREY, E. L. (1935*a*). Growth and glycogen content of the fetal liver and placenta. *Amer. J. Physiol.* **112**, 263–267.

COREY, E. L. (1935*b*). Fetal carbohydrate metabolism following adrenalectomy, insulin and glucose experiments on the mother. *Amer. J. Physiol.* **113**, 450–454.

CORNBLATH, M., LEVIN, E. Y. & MARQUETTI, E. (1958). Studies of carbohydrate metabolism in the newborn. II. The effect of glucagon on the concentration of sugar in capillary blood of the newborn infant. *Pediatrics*, **21**, 885–892.

COWDRY, E. V. (1948). *Laboratory Technique in Biology and Medicine*, 2nd ed., p. 80. Baltimore: The Williams and Wilkins Co.

CRUICKSHANK, E. W. H. (1936). Cardiac metabolism. *Physiol. Rev.* **16**, 597–639.

DAWES, G. S., JACOBSON, H. N., MOTT, J. C. & SHELLEY, H. J. (1960). Some observations on foetal and new-born rhesus monkeys. *J. Physiol.* **152**, 271–298.

DAWES, G. S. & MOTT, J. C. (1959). The increase in oxygen consumption of the lamb after birth. *J. Physiol.* **146**, 295–315.

DAWES, G. S., MOTT, J. C. & SHELLEY, H. J. (1959). The importance of cardiac glycogen for the maintenance of life in foetal lambs and new-born animals during anoxia. *J. Physiol.* **146**, 516–538.

DEMANT, B. (1887). Ueber den Glycogengehalt der Leber neugeborener Hunde. *Hoppe Seyl. Z.* **11**, 142–144.

DESMOND, M. M., HILD, J. R. & GAST, J. H. (1950). The glycemic response of the newborn infant to epinephrine administration: a preliminary report. *J. Pediat.* **37**, 341–350.

FAURÉ-FREMIET, E. & DRAGOIU, J. (1923). Le développement du poumon foetal chez le mouton. *Arch. Anat. micr.* **19**, 411–474.

GELINEO, S. (1954). Le développement ontogenique de la thermoregulation chez le chien. *C.R. Acad. Sci., Paris*, **148**, 1483–1485.

GOLDWATER, W. H. & STETTEN, DE W. JR. (1947). Studies in fetal metabolism. *J. biol. Chem.* **169**, 723–738.

GOODWIN, R. F. W. (1957). The relationship between the concentration of blood sugar and some vital body functions in the new-born pig. *J. Physiol.* **136**, 208–217.

GREGERSEN, M. I., SEAR, H., RAWSON, R. A., SHU CHIEN & SAIGER, G. L. (1959). Cell volume, plasma volume, total blood volume and F_{cells} factor in the rhesus monkey. *Amer. J. Physiol.* **196**, 184–187.

HARD, W. L., REYNOLDS, O. E. & WINBURY, M. (1944). Carbohydrate, fat, and moisture relationships in the pregnant, fetal and newborn guineapig. *J. exp. Zool.* **96**, 189–199.

HITCHCOCK, M. W. S. (1949). Fructose in the sheep foetus. *J. Physiol.* **108**, 117–126.

HUGGETT, A. ST G. (1929). Maternal control of placental glycogen. *J. Physiol.* **67**, 360–371.

HUGGETT, A. ST G. & NIXON, D. A. (1957). Use of glucose oxidase, peroxidase, and *o*-dianisidine in determination of blood and urinary glucose. *Lancet*, **273**, 368–370.

HUGGETT, A. ST G., WARREN, F. L. & WARREN, N. V. (1951). The origin of the blood fructose of the foetal sheep. *J. Physiol.* **113**, 258–275.

JACQUOT, R. (1955). Recherches sur le controle hormonal du glycogène hépatique chez le foetus du rat. *J. Physiol. Path. gen.* **47**, 857–866.

JOST, A. & JACQUOT, R. (1955). Recherches sur les facteurs endocriniens de la change en glycogène du foie foetal chez le lapin (avec des indications sur le glycogène placentaire). *Ann. Endocrin., Paris*, **16**, 849–872.

KEMP, A. & KITS VAN HEIJNINGEN, A. J. M. (1954). A colorimetric micro-method for the determination of glycogen in tissues. *Biochem. J.* **56**, 646–648.

LOCHHEAD, J. & CRAMER, W. (1908). The glycogenic changes in the placenta and the foetus of the pregnant rabbit: a contribution to the chemistry of growth. *Proc. Roy. Soc.* B, **80**, 263–284.

LOVELAND, G., MAURER, E. E. & SNYDER, F. F. (1931). The diminution of the glycogen store of the rabbit placenta during the last third of pregnancy. *Anat. Rec.* **49**, 265–275.

MCCANCE, R. A. & WIDDOWSON, E. M. (1959). The effect of lowering the ambient temperature on the metabolism of the new-born pig. *J. Physiol.* **147**, 124–134.

MARTÍNEK, J. & MIKULÁS, I. (1954). Changes in the glycogen content of muscle after denervation in early post-natal life in rats. *Physiol. Bohemoslov.* **3**, 53–58.

MENDEL, B., KEMP, A. & MYERS, D. K. (1954). A colorimetric micro-method for the determination of glucose. *Biochem. J.* **56**, 639–646.

MENDEL, L. B. & LEAVENWORTH, C. S. (1907). The occurrence of glycogen in the embryo pig. *Amer. J. Physiol.* **20**, 117–126.

MOUNT, L. E. (1959). The metabolic rate of the new-born pig in relation to environmental temperature and to age. *J. Physiol.* **147**, 333–345.

NEEDHAM, J. (1942). *Biochemistry and Morphogenesis*, p. 609. Cambridge University Press.

NEMETH, A. M., INSULL, W. JR. & FLEXNER, L. B. (1954). Glycogenesis in the liver of the fetal guinea pig. *J. biol. Chem.* **208**, 764–772.

PARRY, H. B. & SHELLEY, H. J. (1958). Changes in the liver during the induction of pregnancy toxaemia in sheep. *J. Physiol.* **140**, 48–49*P*.

PFLÜGER, E. (1903). Über den Glykogengehalt der fötalen Leber. *Pflüg. Arch. ges. Physiol.* **95**, 19–22.

RODERICK, L. M., HARSHFIELD, G. S. & HAWN, M. C. (1937). The pathogenesis of ketosis: pregnancy disease of sheep. *J. Amer. vet. med. Ass.* **90**, 41–50.

SCHLOSSMANN, H. (1931). Beiträge zur Biologie der Plazenta. II. Mitteilung: Ist die Plazenta für Insulin durchlässig? *Arch. exp. Path. Pharmakol.* **159**, 213–222.

SCHLOSSMANN, H. (1938). The carbohydrate metabolism of the foetal dog under the influence of insulin. *J. Physiol.* **92**, 219–227.

SHELLEY, H. J. (1960). Tissue carbohydrate reserves in foetal and new-born lambs and rhesus monkeys. *J. Physiol.* **151**, 24–25*P*.

SNYDER, F. F. & HOSKINS, F. M. (1928). The role of the placenta in the carbohydrate metabolism of the foetus. *Anat. Rec.* **38**, 28.

STAFFORD, A. & WEATHERALL, J. A. C. (1960). The survival of new-born rats in nitrogen. *J. Physiol.* **153**, 457–472.

STUART, H. A. & HIGGINS, G. M. (1935). Rhythmic changes in the fetal liver after feeding. *Amer. J. Physiol.* **111**, 590–595.

SZENDI, B. (1934). Über Menge, Verteilung und Bedeutung des Placenta-Glycogens in den verschiedenen Phasen der Schwangerschaft (Beitrag zur Frage der Funktion der menschlichen und Labyrinth–Placenten). *Arch. Gynaek.* **158**, 409–425.

SZENDI, B. (1936). Intrauterine Funktion der Lunge und Leber des Fetus. *Arch. Gynaek.* **162**, 27–41.

TAYLOR, P. (1960). The influence of environmental temperature on the O_2 consumption of new-born rats. *J. Physiol.* **151**, 22–23*P*.

VILLEE, C. A. (1953*a*). Biochemical differentiation of human fetal tissues with age. In *Prematurity, Congenital Malformation and Birth Injury*, pp. 78–83. New York: Association for the Aid of Crippled Children.

VILLEE, C. A. (1953*b*). Regulation of blood glucose in the human fetus. *J. appl. Physiol.* **5**, 437–444.

VILLEE, C. A. (1954). The intermediary metabolism of human fetal tissues. *Cold Spr. Harb. Symp. quant. Biol.* **19**, 186–199.

WALLACE, L. R. (1948). The growth of lambs before and after birth in relation to the level of nutrition. Part II. *J. Agric. Sci.* **38**, 243–302.

J. Physiol. (1960), **153**, *pp.* 553–561
With 3 text-figures
Printed in Great Britain

THE EFFECT OF OXYTOCIN ON THE URINARY EXCRETION OF WATER AND ELECTROLYTES IN MAN

BY R. B. CROSS, S. E. DICKER, A. H. KITCHIN, SYBIL LLOYD AND MARY PICKFORD

Departments of Pharmacology, University College London, and of Physiology, University of Edinburgh

(*Received* 18 *May* 1960)

In some animals the administration of posterior pituitary extracts produces both a chloruretic and natriuretic effect (Fraser, 1937, 1942; Heller & Stephenson, 1950) and modifies renal haemodynamics (Dicker & Heller, 1946). The question arises whether these effects are due to the antidiuretic or oxytocic fraction. Whereas there is good evidence to show that in the rat the oxytocic and not the antidiuretic fraction enhances the excretion of these ions, especially during saline diuresis (Kuschinsky & Bundschuh, 1939; Fraser, 1942; Dicker & Heller, 1946; Schaumann, 1949; Heller & Stephenson, 1950; Brunner, Kuschinsky & Peters, 1956), in the dog both fractions are active. Thus, Sartorius & Roberts (1949) and Anslow & Wesson (1955) showed that vasopressin regularly enhanced Na and Cl excretion during water diuresis, and occasionally also at low rates of flow. Brooks & Pickford (1958) confirmed these findings regarding the antidiuretic hormone and further showed that the oxytocic fraction consistently increased electrolyte excretion at low rates of urine flow. Regarding the renal vascular effects of the hormones, Demunbrun, Keller, Levkoff & Purser (1954) reported that the intravenous infusion of vasopressin in dogs suffering from diabetes insipidus caused by extensive hypothalamic lesions did not affect the low glomerular filtration rate and renal plasma flow encountered, whereas the administration of oxytocin restored renal haemodynamics to preoperative levels.

There is relatively little information as to the effects of posterior pituitary extracts on renal function in man. Chalmers, Lewis & Pawan (1951) injected human subjects with doses of 50–200 m-u. Pitocin and 20–100 m-u. Pitressin and found that neither of these preparations had an effect on the excretion of Na or Cl. This was confirmed by Thomson (1960) using a synthetic oxytocin preparation. No information, however, is available as to a possible action of oxytocin on glomerular filtration rate or renal plasma flow, nor whether it affects electrolyte excretion during saline diuresis. It was therefore decided to investigate further the action of oxytocin in man,

using both the commercial extract of posterior pituitary lobe, and a synthetic oxytocin preparation whose actions cannot be differentiated from those of highly purified natural oxytocin as prepared by du Vigneaud and his colleagues (Brooks & Pickford, 1958).

METHODS

The subjects were twenty men of 19–60 years of age and fifteen women of 25–55 years. Nine of the women had had a normal labour in the Obstetric Unit of University College Hospital, London, at least 10 days before the experiments were made. All experiments were performed with the subjects recumbent. In all but three of the women urine was collected through a catheter inserted before the beginning of the experiment. The catheter drained the urine directly into a measuring cylinder under the bed. The men and three of the women stood up for micturition. In experiments where the effect of oxytocin was observed during water diuresis, 1–2 l. of water was swallowed and the water load kept constant in most of the experiments by replacing the volume of urine excreted by equal amounts of ingested water. In other experiments, when the subjects took no doses of water, the effect of oxytocin was tested at low rates of urine flow.

The effect of oxytocin was also observed after the subject had been loaded with sodium chloride. On seven occasions the drug was infused during 'saline' diuresis induced by the ingestion of 700–800 ml. of a 0·9 % NaCl solution; on two occasions during water diuresis which resulted from 1000 ml. water taken 4 hr after a normal breakfast to which salt had been added to the limit of toleration; and on five occasions during water diuresis after a lunch made as salty as possible, with a further 5 g NaCl given 2 hr later, immediately before the dose of water. Lastly, since Ladd (1952) observed that prehydration 8–13 hr before intravenous infusion of 0·9 % NaCl solution profoundly affected the response of the kidney to the infusion, three experiments similar to but not identical with his were performed. Three subjects drank 2–2·5 l. of water in the evening between 10·30 and 11·30 p.m., and next morning at 9 a.m. a 'saline' diuresis was induced by drinking 700–800 ml. of a 0·9 % NaCl solution. Since all these experiments gave similar results, they will be grouped together under the heading of salt 'loading'.

Infusions were made intravenously with either a 5 % glucose or a 0·9 % NaCl solution given by motor-driven syringe delivering at a constant speed of 0·18 or 0·75 ml./min. To avoid psychological disturbances which might affect the results two methods were used. Either control and experimental infusions were run on different days without informing the subject which was being used, or the needle was inserted into the vein and the control solution run for some time before the drug infusion was begun. Test substances were either given at the peak of diuresis or when the rate of urine flow was steady.

Glomerular filtration rate (G.F.R.) and renal plasma flow (R.P.F.) were estimated in terms of insulin and *p*-amino hippuric acid (PAH) clearances. Following an intravenous priming injection of 100 ml. of a 0·9 % NaCl solution containing 5 g inulin and 0·8 g PAH, the subjects received a sustaining infusion of 65 mg inulin and 6 mg PAH/min in a 0·9 % NaCl solution at a rate of 1·0 or 1·5 ml./min. Test substances were diluted in the infusion mixture and the change from control to test infusion was made without the knowledge of the subject. No urine collections were made until 45–60 min. after the priming injection. On a number of occasions the endogenous creatinine clearance was measured instead of that of inulin.

Creatinine was estimated by the alkaline picrate method, inulin by Cole's unpublished modification of the method of Hubbard & Loomis (1942) in which $FeCl_3$ is used as a catalyst and PAH by the method of Smith, Finkelstein, Aliminosa, Crawford & Graber (1945). Na and K were estimated by flame photometry, and Cl by the method of Prout-Winter as described by Cole (1919).

The drugs used were the synthetic oxytocin, Syntocinon (Sandoz Ltd.) and the posterior pituitary extracts, Pitocin and Pitressin (Parke, Davis and Co.). Since the synthetic and extracted oxytocins had identical effects the term oxytocin will be used.

RESULTS

Effect of oxytocin in the absence of salt loading. A total of twenty-four experiments was performed at various rates of urine flow, depending on whether water was ingested. The lowest rate of urine flow was 0·54 ml./min and the highest 18 ml./min. The rates at which oxytocin was infused were 4, 8, 16, 32, 64, 120, 150 and 200 m-u./min. It may be seen in Fig. 1 that an

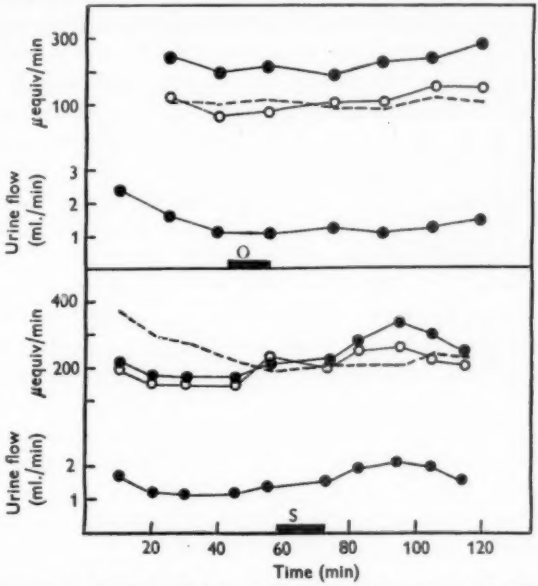


Fig. 1. Comparison of effect of intravenous infusion of oxytocin and of 0·9 % NaCl solution in the same subject. Lower curve in both frames shows rate of urine flow; upper curves in both frames show rate of excretion of Na (●), K (○) and Cl (---). Blocks on abscissae show duration of infusion of oxytocin 200 m-u./min (O in upper frame) and of 0·9 % NaCl solution (S in lower frame). Water load maintained constant by intake of that volume of fluid excreted in urine.

infusion of as much as 200 m-u./min had no more effect on water, Na, K and Cl excretion than the infusion of 0·9 % NaCl solution under closely similar conditions and on the same subject. Negative results with regard to electrolyte excretion were obtained whatever the rate of urine flow in all but one subject who showed during water diuresis a long-lasting rise in Na excretion beginning 10–20 min after the end of the infusion; further observation, however, showed that in this subject a similar rise occurred after a

control infusion of a 5% glucose solution. Clearly, then, changes in electrolyte excretion do not follow the administration of oxytocin in the circumstances considered. Oxytocin did not affect the rate of urine flow except in one subject who was observed on three different occasions. During water diuresis an infusion of 150 m-u. oxytocin/min for 12 min had no effect on the rate of urine flow. However, twice in the absence of hydration the same amount of oxytocin had a clear antidiuretic effect: on one of these occasions the inhibition was so pronounced and long-lasting that the experiment had to be abandoned; on the other occasion, the urine flow fell from 2·3 to 1·0 ml./min with a recovery to 1·4 ml./min after 40 min. In contrast, oxytocin was apparently diuretic once only out of several occasions in one subject: on the exceptional day he was not hydrated and was excreting urine at the rate of 2·8 ml./min; immediately following a 15 min infusion of 150 ml./min oxytocin the rate of flow increased to 8·0 ml./min; this fell to 4·0 ml./min an hour later. No change occurred in the excretion of either Na or K.

Effect of oxytocin after salt loading. A total of twenty-five experiments and their controls were performed on fourteen subjects in one of the circumstances described as 'salt loading' under Methods. In all instances where the rate of urine flow was high or moderately high, infusion of oxytocin at rates ranging from 16 to 200 m-u./min had no more effect on the excretion of electrolytes than the infusion of control solutions of glucose or NaCl. The results were also negative in four out of five experiments performed at low rates of urine flow. The apparently positive result was obtained in the first of the exceptional subjects mentioned in the previous section; after salt loading as in water diuresis, about 30 min after the end of the infusion this man showed a steady hour-long rise in Na excretion and no change in K excretion. He showed a similar increase in Na excretion after being infused with 5% glucose solution only, so that the enhanced natriuresis could not be attributed to the action of oxytocin. The rate of urine flow in saline diuresis was unaffected by oxytocin except on two occasions in the subject mentioned previously who showed an inhibition when unhydrated; thus, an infusion of 100 m-u./min oxytocin for 27 min reduced his rate of urine flow from 11·9 to 1·7 ml./min, with recovery to 5·2 ml./min 60 min after the end of the infusion.

Effect of oxytocin combined with vasopressin. It has been shown in rats and dogs that one or more of the renal actions of oxytocin are decreased by the simultaneous administration of vasopressin (Fraser, 1942; Brooks & Pickford, 1958). To test whether this held true in humans the effect of infusion of 1·0 m-u. vasopressin/min for 30 min was compared in the same subjects, either on the same day or a few days later, with the effect of infusion of a mixture of the same amount of the drug with two to fifty

times more oxytocin. The subjects were nine healthy post-parturient women and the observations were made during water diuresis. In no instance was the antidiuretic response following the infusion of vassopressin alone different from that seen during infusion of a mixture of vasopressin and oxytocin (Fig. 2).

One of the women was accustomed to smoking about fifteen cigarettes a day. The vigorous and rapid smoking of two cigarettes consecutively at the peak of a water diuresis resulted in an antidiuresis which was quantitatively similar to that observed from the infusion of 2·0 m-u. Pitressin/min. The antidiuretic effect was reproducible from day to day and was found to be unaffected by the simultaneous infusion of 16 m-u. oxytocin/min.

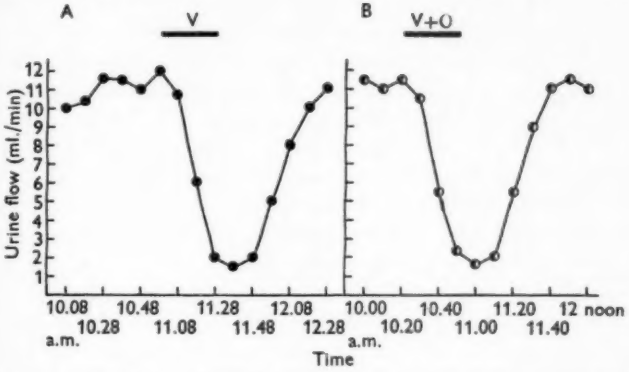


Fig. 2. Comparison of the effect of intravenous infusions of vasopressin and of a mixture of vasopressin and oxytocin on the urinary flow in the same subject. In A, the subject was infused with vasopressin at a rate of 1·0 m-u./min. In B, 3 days later, the subject was infused with the same amount of vasopressin mixed with fifty times more oxytocin. Duration of infusions shown by the blocks. In both experiments the water load of 900 ml. water was kept constant by intake of volumes of water equal to those excreted in urine.

Effect of oxytocin on renal clearances. Renal clearances of endogenous creatinine were measured twice during water diuresis, twice during 'saline' diuresis and once at low rate of urine flow due to absence of hydration. The clearance values, however, varied so much during the course of any one set of observations that it was impossible to draw any conclusions from the results. In all other cases values for G.F.R. and R.P.F. were estimated by inulin and PAH clearances. Seven observations were made on five male subjects: they were given doses of oxytocin ranging from 100 to 750 m-u./min for periods of time varying from 10 to 30 min during the sustaining infusion of inulin and PAH. Twice when 150 m-u./min oxytocin was infused, and once when 200 m-u./min was given, the renal clearances of

inulin and PAH were unaffected. On the other hand, Fig. 3 shows an experiment in which G.F.R. and R.P.F. appeared to be decreased by only 100 m-u. oxytocin/min for 33 min, and greater falls were obtained on one occasion each with infusions of 140, 200, and 750 m-u./min.

In all these experiments oxytocin had an antidiuretic effect when given during infusions of inulin and PAH. For instance, a subject who showed no antidiuretic response to 200 m-u./min oxytocin during water diuresis

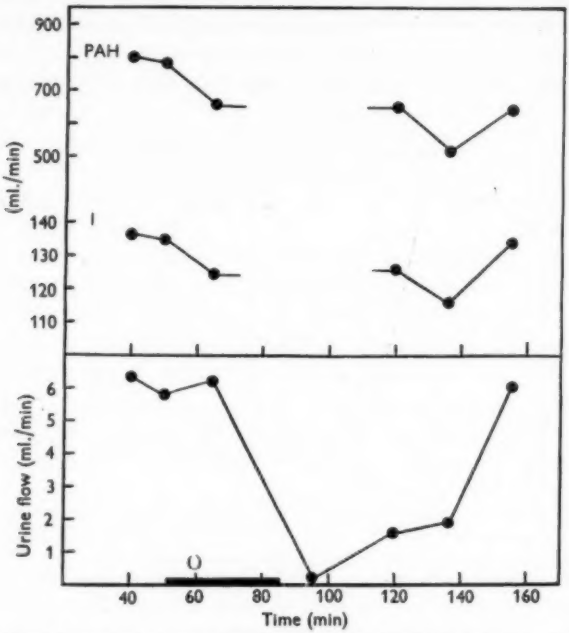


Fig. 3. Effect of oxytocin on rate of urine flow (lower frame) and clearances of PAH and inulin (upper frame). Zero time taken as the time when sustaining intravenous infusion of PAH and inulin was begun. Block marked O indicates time during which 100 m-u./min oxytocin was added to the sustaining infusion. Water load maintained constant by intake of that volume of fluid excreted in urine.

(Fig. 1) or on two occasions during 'saline' diuresis, had his diuresis markedly inhibited by the same dose of oxytocin given for 17 min during the simultaneous infusion of inulin and PAH: the rate of urine flow fell from 1·2·5 to 1·7 ml./min for 45 min and thereafter increased to 2·5 ml./min. In another subject previously unaffected by an infusion of 100 m-u. oxytocin/min the same dose given during clearance measurements caused a marked antidiuresis (Fig. 3). In fact, in all subjects the administration of oxytocin during inulin and PAH clearance estimations produced some

antidiuresis, even in those instances where changes in clearance values were insignificantly small, as in the example shown in Fig. 3.

DISCUSSION

The present results show that the effects of oxytocin in human beings differ from those observed in other species in at least three aspects. First, in dogs oxytocin raises the urinary output of Na and Cl, especially when administered at low rates of urine flow (Brooks & Pickford, 1958). Similar effects have been observed in rats (Dicker & Heller, 1946; Heller & Stephenson, 1950; Croxatto, Rosas & Barnafi, 1956; Brunner *et al.* 1956). In human beings, however, infusions of oxytocin had no effect on Na, K or Cl excretion over a wide range of urine concentration and rate of flow; this agrees with Chalmers *et al.* (1951) and Thomson's (1960) findings. Secondly, in dogs the intravenous administration of oxytocin always produces a marked increase in R.P.F., without necessarily a simultaneous change in G.F.R. (Brooks & Pickford, 1958; Ali, 1958). Similarly in rats, oxytocin increases both inulin and diodone clearances (Dicker & Heller, 1946) though the clearance of endogenous creatinine is unaffected (Brunner, Kuschinsky, Munchow & Peters, 1957). In contrast, in man, when fairly large doses of oxytocin were infused, on four occasions out of a total of seven experiments there was a *decrease* of both G.F.R. and R.P.F. No increase was ever observed. As further observations, however, showed that oxytocin is antidiuretic in the presence of inulin and PAH, the validity of the clearance values may be questioned. The possibility that oxytocin might not affect renal haemodynamics, but for the presence of the test substances used for their measurement, must be borne in mind. Thirdly, the effect of oxytocin on the rate of urine flow is not the same in all species. Croxatto *et al.* (1956) and Brunner *et al.* (1957) observed an increase of urine flow in the rat, especially in conditions where the urine was concentrated, whereas van Dyke, Adamson & Engel (1955) and Brooks & Pickford (1958) found oxytocin to have an antidiuretic action in the dog. In man the results to date are conflicting. From the present findings, in twenty-seven out of twenty-nine experiments in recumbent subjects oxytocin infused *alone*, during either water or 'saline' diuresis, had no antidiuretic effect and on one occasion even produced a diuretic response. Thomson (1960), however, observed an antidiuretic effect in fifteen out of seventeen experiments, using doses of oxytocin in the same range as those used in the present investigation. The only obvious difference in the technique used by Thomson and the present authors is that in Thomson's experiments subjects were sitting, whereas in the present investigation they were all lying down. The importance of the posture of the subjects is borne out by

Chalmers *et al.* (1951) observations on men in whom oxytocin was moderately antidiuretic in seven out of eight cases. Though it is not stated in their paper, Chalmers in a personal communication informed us that all his subjects were either seated or ambulant. It is therefore conceivable that the upright posture may affect the renal vascular responses, which in turn will determine the presence or the absence of antidiuresis.

The reasons for these species differences are not known. The interpretation of these results will remain difficult until more is known about the physiological level of circulating oxytocin in animals as well as in man.

SUMMARY

1. The effect of intravenous infusions of synthetic oxytocin on the excretion of water, Na, K and Cl was investigated in recumbent subjects of both sexes. The doses of oxytocin infused varied from 4 to 200 m-u./min. Observations were made when the rate of urine flow was either low or high, and after the subjects had been loaded with NaCl.

2. With the exception of one male subject, oxytocin did not produce any antidiuresis. In all subjects the excretion of Na, K and Cl was not more affected by oxytocin than by control infusions of either 5% glucose or 0·9% NaCl solutions.

3. The infusion of 1 or 2 m-u. vasopressin/min together with fifty times as much oxytocin had the same effect as that to be expected had vasopressin been given alone.

4. In four out of seven observations oxytocin in doses from 100 to 750 m-u./min caused some fall in the clearances of both inulin and PAH.

The authors wish to express their thanks to all patients, colleagues and students who volunteered to act as subjects for this investigation. One of us (S.E.D.) is indebted to Professor W. C. W. Nixon for allowing part of this investigation to be carried out in University College Obstetric Hospital.

REFERENCES

Ali, M. N. (1958). A comparison of some activities of arginine vasopressin and lysine vasopressin on kidney function in conscious dogs. *Brit. J. Pharmacol.* **13**, 131–137.

Anslow, W. P. & Wesson, L. G. (1955). Some effects of pressor-antidiuretic and oxytocic fractions of posterior pituitary extracts on sodium, potassium and ammonium excretion in the dog. *Amer. J. Physiol.* **182**, 516–566.

Brooks, F. P. & Pickford, M. (1958). The effect of posterior pituitary hormones on the excretion of electrolytes in dogs. *J. Physiol.* **142**, 468–493.

Brunner, H., Kuschinsky, G., Münchow, O. & Peters, G. (1957). Der Einfluss von natürlichem und synthetischem Oxytocin auf endogene Kreatinin-Clearance, Salzausscheidung und Säureausscheidungfähigkeit der Ratte und auf die Diurese des Menschen. *Arch. exp. Path. Pharmak.* **230**, 80–89.

Brunner, H., Kuschinsky, G. & Peters, G. (1956). Der Einfluss von Oxytocin auf die renale Wasser- und Salzausscheidung der Ratte. *Arch. exp. Path. Pharmak.* **228**, 457–473.

Chalmers, T. M., Lewis, A. A. G. & Pawan, G. L. S. (1951). The effect of posterior pituitary extracts on the renal excretion of sodium and chloride in man. *J. Physiol.* **112**, 238–242.

Cole, S. W. (1919). *Practical Physiological Chemistry.* Cambridge: W. Heffer and Sons, Ltd.

CROXATTO, H., ROSAS, R. & BARNAFI, L. (1956). The effect of vasopressin and oxytocin on the excretion of water and electrolytes (Na, Cl and K) in the albino rat. *Acta physiol. latinoamer*, **6**, 147–152.

DEMUNBRUN, T. W., KELLER, A. D., LEVKOFF, A. F. & PURSER, R. M. (1954). Pitocin restoration of renal hemodynamics to pre-neurohypophysectomy levels. *Amer. J. Physiol.* **179**, 429–434.

DICKER, S. E. & HELLER, H. (1946). The renal action of posterior pituitary extract and its fractions as analysed by clearance experiments on rats. *J. Physiol.* **104**, 353–360.

FRASER, A. M. (1937). The diuretic action of the oxytocic hormone of the pituitary gland and its effect on the assay of pituitary extracts. *J. Pharmacol.* **60**, 89–95.

FRASER, A. M. (1942). The action of the oxytocic hormone of the pituitary gland on urine secretion. *J. Physiol.* **101**, 236–251.

HELLER, H. & STEPHENSON, R. P. (1950). Effect of posterior pituitary extract and its fractions on renal electrolyte excretion. *Nature, Lond.*, **165**, 189.

HUBBARD, R. S. & LOOMIS, T. A. (1942). The determinatiou of inulin. *J. biol. Chem.* **145**, 641–645.

KUSCHINSKY, G. & BUNDSCHUH, H. E. (1939). Über eine diuretische und Kochsalz-ausschwemmende Substanz in Hypophysenhinterlappen-Präparaten. *Arch. exp. Path. Pharmak.* **192**, 683–700.

LADD, M. (1952). Renal excretion of sodium and water in man as affected by prehydration, saline infusion, pitressin and thiomerin. *J. appl. Physiol.* **4**, 602–619.

SARTORIUS, O. W. & ROBERTS, K. (1949). The effects of Pitressin and desoxycorticosterone in low dosage on the excretion of Na, K and water by the normal dog. *Endocrinology*, **45**, 273–283.

SCHAUMANN, O. (1949). Über den Einfluss von Oxytocin und Vasopressin auf die Ausscheidung von Kalium und Natrium durch die Niere. *Experientia*, **5**, 360–361.

SMITH, H. W., FINKELSTEIN, N., ALIMINOSA, L., CRAWFORD, B. & GRABER, M. (1945). The renal clearances of substituted hippuric acid derivatives and other aromatic acids in dogs and man. *J. clin. Invest.* **24**, 388–404.

THOMSON, W. B. (1960). The effect of oxytocin and vasopressin and of phenylalanin-oxytocin on the urinary excretion of water and electrolytes in man. *J. Physiol.* **150**, 284–294.

VAN DYKE, H. B., ADAMSON, K. & ENGEL, S. L. (1955). Aspects of the biochemistry and physiology of the neurohypophyseal hormones. *Recent Progr. Hormone Res.* **11**, 1–35.

J. Physiol. (1960), **153**, *pp.* 562–572
With 3 text-figures
Printed in Great Britain

DIFFUSION OF ACETYLCHOLINE IN AGAR GELS AND IN THE ISOLATED RAT DIAPHRAGM

BY K. KRNJEVIĆ AND J. F. MITCHELL

From the Agricultural Research Council Institute of Animal Physiology, Babraham, Cambridge

(*Received* 20 *May* 1960)

In the course of experiments in which acetylcholine (ACh) released during nerve stimulation was collected from the isolated rat diaphragm (Krnjević & Mitchell, 1960), it became a matter of some importance to know how ACh is distributed within the diaphragm and at what rate it can be expected to diffuse out. The following experiments were therefore undertaken, and, as no information was available about the rate of diffusion of ACh in physiological solutions, estimations were also made of the diffusion coefficient of ACh in gels of Ringer–Locke solution and agar.

METHODS

Diffusion of ACh chloride in agar gels

The method used was derived from one described by Eggleton, Eggleton & Hill (1928) in which measurements were made of the amounts of substances diffusing out of an agar gel. In our first experiments the solution of ACh chloride and agar was poured into a boiling-tube (cross-section about 4 cm^2) and allowed to set, and the rate of diffusion of ACh into physiological saline determined exactly as in the experiments of Eggleton *et al.* (1928). The apparent coefficients of diffusion obtained in this way were unexpectedly high, and, for control, measurements of the diffusion of NaCl into water were made under the same conditions. These also showed very high apparent rates of diffusion (as much as 50 % higher than the values given in the International Critical Tables). Inspection of the diffusion tubes showed that this anomalous diffusion could be ascribed to a substantially greater (+ 10–20 %) area of diffusion at the curved surface of the gel than would be expected from the area of cross-section of the tube. The method was therefore modified as follows.

'Bacto-agar' (Difco Laboratories, Detroit, Michigan) was dissolved in hot Ringer–Locke solution so as to make a 0·5 % (w/v) solution. It was allowed to cool to about 40° C, at which temperature it remained liquid. ACh chloride was then added from a standard solution to give a final concentration in the agar solution of $5{\cdot}0 \times 10^{-5}$ M, the standard solution (5×10^{-3} M) being prepared from a known weight of acetylcholine chloride (Roche Products) dissolved in a solution of 0·1 M-NaH_2PO_4. The Ringer–Locke solution had the following composition (mM): Na^+ 150, K^+ 5·0, Ca^{2+} 2·0, Mg^{2+} 1·0, Cl^- 148, $H_2PO_4^-$ 1·0, HCO_3^- 12·0 and glucose 11, the ionic strength being 0·164.

Diffusion chamber. This consisted of two Perspex tubes. One, with an internal diameter of about 3 cm and length of about 5 cm, was closed at one end; the other (about 4 cm long) was open at both ends and fitted accurately over the open end of the first. Some 25 ml. of the warm solution of ACh and agar was poured carefully into the bottom tube so as to give

a very smooth and flat surface at the open end. When the agar gel had set and reached the desired temperature (by equilibration with a large volume of water in which the chamber was immersed), the upper tube was fitted on. 20·0 ml. portions of Ringer–Locke solution were pipetted into the upper compartment, in contact with the surface of the gel, and kept stirred by bubbles of moist compressed air. They were replaced at intervals so that the external ACh concentration never rose above 1–2 % of that in the agar gel. The amounts of ACh collected were assayed in the conventional manner on a frog rectus preparation, using for comparison dilute solutions of ACh prepared from the same standard. The accuracy of the method of assay was estimated by including ACh solutions of a known concentration in each batch of 'unknowns'. The ACh content of a series of ten such solutions was estimated with a coefficient of variation of $\pm 6{\cdot}2\,\%$. There was also a systematic tendency to underestimate the apparent content by an average of 2·5 %. With this modified method, an estimate of the rate of diffusion of NaCl came to within 5 % of the expected values.

Diffusion of ACh through diaphragm muscle

Rats weighing 300–400 g were anaesthetized with ether, and their entire diaphragms were then removed in one piece, still attached to the costal margin. After soaking in cold Ringer–Locke fluid, well aerated with 5 % CO_2 in O_2, the muscular portion of one half of the diaphragm was stretched lightly and tied over the smooth end of a hollow Perspex cylinder (internal diameter 1·28 cm). Excess tissue was trimmed off, and the cylinder, occluded by the disk of diaphragm muscle, was dipped into a small beaker containing 5·0 ml. of Ringer–Locke fluid kept agitated by bubbles of 5 % CO_2 in O_2. A solution of ACh chloride in Ringer–Locke fluid (which varied between 5×10^{-5} and 5×10^{-4} M) was pipetted into the lumen of the cylinder until the fluid level inside equalled that in the beaker outside. A similar gas mixture stirred the internal solution (volume about 1·5 ml.), which was replaced by fresh ACh solution every 2–5 min. The external solution was changed at intervals of 10–15 min, allowing a sufficient amount of ACh to diffuse through to give an adequate concentration for assay. To prevent destruction of ACh by cholinesterases in the tissue, eserine sulphate was added to the solutions ($1{\cdot}5 \times 10^{-5}$ M); alternatively, the rats before death were given intraperitoneal injections of di-isopropylphosphorofluoridate (DFP) in doses of 5 μmole/100 g body weight.

Estimation of thickness of diaphragms. 20 μ frozen sections of the muscles used in diffusion studies were cut and examined immediately, either unstained or after light colouring with haematoxylin and eosin.

ACh space within the diaphragm muscle. The diaphragms were removed from 300 g rats anaesthetized with ether and, after a preliminary 20–30 min period of soaking in cold Ringer–Locke solution well aerated with 5 % CO_2 in O_2, the muscles were dissected free from ribs and connective tissue, lightly blotted with filter paper and quickly weighed (usual weight 200–300 mg). They were allowed to soak in well-aerated and well-stirred Ringer–Locke fluid, containing 5×10^{-4} M ACh chloride, for periods of some 60–120 min; after this they were blotted with filter paper as before, and transferred to a beaker containing 5·0 ml. of well-stirred Ringer–Locke solution. 1-ml. samples of this solution were removed for assay after set intervals, the volume of Ringer–Locke fluid being maintained approximately constant by the addition of known amounts of fresh solution from time to time as required.

In the first experiments of this kind destruction of ACh was prevented by eserine sulphate ($1{\cdot}5 \times 10^{-5}$ M); in later experiments the rats were given an intraperitoneal injection of DFP (5 μmole/100 g body weight). A further modification in later experiments was that the diaphragms were not cut away from the ribs until *after* the period of soaking in ACh. A number of samples were treated with strong alkali (1/5th volume of N/3-NaOH) and then neutralized to exclude the possibility of a release of an interfering substance from the muscle.

RESULTS

Diffusion of ACh from a Ringer–Locke-agar gel into Ringer–Locke solution

When diffusion takes place from a semi-infinite source into a well-stirred solution in which the concentration is maintained at zero, the amount of substance diffusing out is proportional to the square root of time. It can be shown (Stefan, 1871; Crank, 1956) that the total quantity (Q) which has diffused out after time t should be

$$Q = 2AC\sqrt{(Dt/\pi)}, \quad (1)$$

where A is the area across which diffusion takes place, C the initial uniform concentration in the source, and D the appropriate diffusion coefficient.

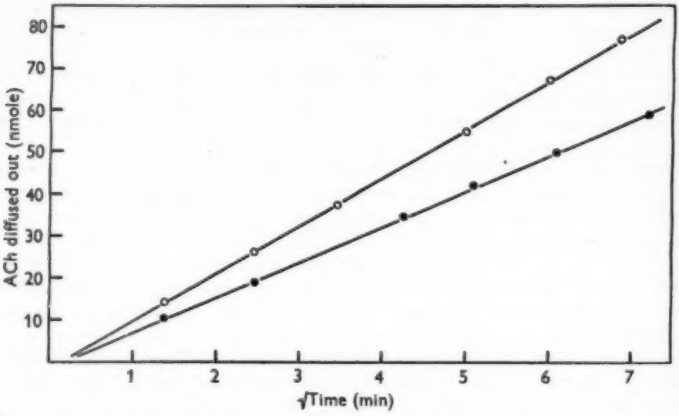


Fig. 1. Diffusion of ACh from a Ringer–Locke-agar gel into well-stirred Ringer–Locke solution. Ordinates indicate the total amount of ACh that had left the gel at a given time. Straight lines were drawn by eye, through points obtained during one experiment at 21° C (●) and another at 37·5° C (○).

In practice, finite systems behave as semi-infinite sources so long as the concentration does not fall appreciably below C at some distance from the boundary. For a system which is several centimetres deep, this condition would be expected to hold for many hours, but in any case it was advisable to find out whether the simple relation between Q and $\sqrt{t}$ really held before making further use of this equation.

We found in our experiments that plots of Q against $\sqrt{t}$ gave good straight lines in the time during which observations were made (up to 2 hr). Two examples of such graphs are shown in Fig. 1. There were small but systematic differences between observations made on different occasions,

no doubt because of unavoidable small variations in such factors as the area of diffusion, the concentration of ACh, and the temperature. In all cases, however, there was a linear relation between Q and $\sqrt{t}$, which justified the use of Equation (1) for determining the coefficient of diffusion (D); and the only feature worthy of comment is that, at room temperature, there was a consistent tendency for the straight lines to cut the abscissae a short distance on the positive side of zero (as in Fig. 1).

Diffusion at room temperature. In all, eleven experiments were performed at room temperature (20, range $\pm 1°$ C) using the same diffusion chamber (with a diffusion area of 6·80 cm²), and the same concentration of acetylcholine chloride (5×10^{-5} M); from these experiments 58 pairs of values of Q and $\sqrt{t}$ were obtained. These values were all grouped together, and a regression coefficient was calculated for the regression of Q on $\sqrt{t}$. The line of best fit so obtained is described by Equation (2), where $\sqrt{t}$ is given in $\text{min}^{\frac{1}{2}}$ and Q in nmole:

$$Q = 9{\cdot}32\sqrt{t} - 3{\cdot}54, \tag{2}$$

the S.E. of the coefficient being $\pm 0{\cdot}20$. From this relation between Q and $\sqrt{t}$, the diffusion coefficient in Ringer–Locke solution was calculated to be $9{\cdot}8(\pm 0{\cdot}42) \times 10^{-6}$ cm²/sec, for $20 \pm 1°$ C.

Diffusion at 36·5° C. For comparison, four experiments were performed under similar conditions but at 'body' temperature, i.e. $36{\cdot}5 \pm 1{\cdot}5°$ C. Twenty-one pairs of values of Q and $\sqrt{t}$ gave a line described by the following equation:

$$Q = 11{\cdot}44\sqrt{t} + 0{\cdot}95, \tag{3}$$

in which the regression coefficient had a S.E. of $\pm 0{\cdot}36$. The consequent value for the diffusion coefficient in Ringer–Locke solution at $36{\cdot}5 \pm 1{\cdot}5°$ C was $14{\cdot}8\ (\pm 0{\cdot}93) \times 10^{-6}$ cm²/sec.

Diffusion of ACh through the rat diaphragm

Four experiments were performed, special attention being paid to the steady-state conditions rather than to the kinetics of diffusion. The aim was to find the apparent coefficient of diffusion on the assumption that the entire surface of the diaphragm exposed to the ACh solution was available for diffusion. The coefficient was calculated from

$$Q = AD' \frac{dc}{dx}, \tag{4}$$

where Q is the amount of ACh diffusing per unit time, dc/dx the uniform gradient of concentration across the diaphragm, A the area of the muscle exposed (1·29 cm²), and D' the apparent diffusion coefficient. The concentration of ACh in the solution on the upper surface of the muscle (C_0)

varied between 5×10^{-5} and 5×10^{-4} M in different experiments; the ACh concentration in the external solution, in contact with the lower surface, was not allowed to rise above 1–2 % of C_0. It was assumed that during steady-state diffusion the gradient of concentration was C_0/L, L being the thickness of the diaphragm measured in frozen sections (see below). Q was estimated by assaying samples of the external solution for ACh. Q appeared to reach a steady level within 30 min, and although there were small variations after that, they did not exceed 10–25 % over a further period of 1–3 hr. For calculating D', values of Q were averaged out over a period of at least 1 hr, beginning 30 min after the start of the experiment. The resulting values of D' ranged from 1·15 to $1{\cdot}6 \times 10^{-6}$ cm²/sec, with a mean of $1{\cdot}4 \times 10^{-6}$ cm²/sec (at a temperature of $20 \pm 1°$ C).

Thickness of rat diaphragms. Seventeen measurements were made of the thickness of diaphragms from six rats, all of body weight about 300 g. There was as much variation of thickness within one muscle as there was between different diaphragms. The mean thickness in frozen sections was 0·94 mm, with an S.D. of ± 0·036 mm.

Kinetics of diffusion of ACh from the diaphragm

The outward movement of ACh (at $20 \pm 1°$ C) from diaphragms previously equilibrated for 1–2 hr with 5×10^{-4} M ACh is shown on a semi-log. scale in Figs. 2 and 3. The four muscles of Fig. 3 had been cut from the ribs before soaking in ACh, whereas the five muscles of Fig. 2 had not. In both groups a straight line was drawn through the later, slow phase of outward movement and extrapolated to zero time. It seems that a certain proportion of the ACh in the muscle was unable to diffuse freely, either because it had entered muscle fibres, or because it was bound to some tissue component. In muscles not cut away from the ribs before soaking in ACh, the fraction of 'slow' ACh amounted to about 0·15; in those muscles which were cut away from the ribs the fraction was about 0·38. The initial phase of quick outward movement of ACh is also shown in each figure on an expanded time scale. These points were determined by subtraction of the slow component, and a theoretical diffusion curve for a plane sheet was fitted to the points of Fig. 2 by using appropriate values of Dt/l^2 (where $2l$ is the thickness of the tissue) given by Hill (1928, Fig. 5). The same curve was also drawn through the fast points in Fig. 3; although it fitted only approximately, this was about as good a fit as could be obtained (for any value of D/l^2) with the diffusion equation for a plane sheet, presumably because of the distortion caused by diffusion from the cut end of the fibres. It may be noted that one half of the 'free' ACh was lost in about 1·5 min, and that the later, straight portion of this diffusion curve had a half-time of 2·4 min. If one takes the value of $9{\cdot}8 \times 10^{-6}$ cm²/sec for D, which was

obtained as already described, the apparent value of l (the diffusion distance) is 1·4 mm.

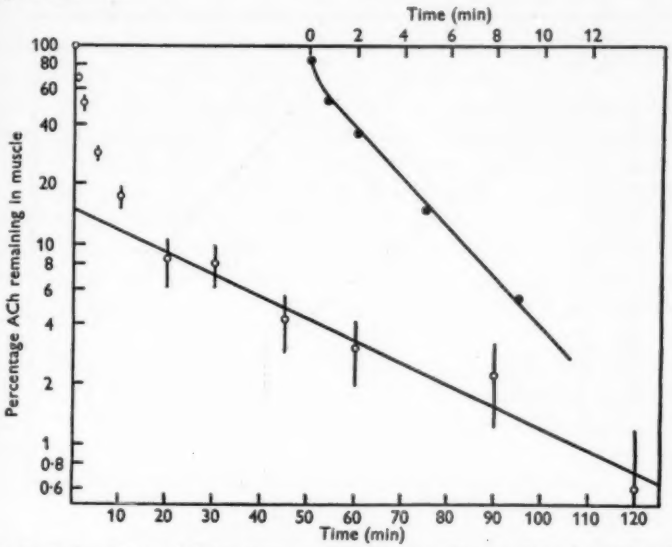


Fig. 2. Rate of loss of ACh from rat diaphragms after prolonged soaking in $5–10^{-4}$ M ACh. Each point (○) is mean of 5 observations and vertical lines show $\pm 1 \times$ S.E. Straight line was drawn by eye through last six points. Fast initial component (●) was obtained by subtraction of straight line, and is shown on expanded time scale in upper right quadrant. A diffusion curve was fitted to these points by using value of 0·002 for D/l^2. Semi-log. scale.

ACh space inside the diaphragm

After soaking diaphragms in 5×10^{-4} M ACh chloride for 1–2 hr at about 20° C, the ACh diffused out into an ACh-free solution as shown above. In our initial experiments we relied on eserine to prevent destruction of ACh, but found that an appreciable amount of ACh was being lost. We therefore injected DFP into the rats before removing the diaphragms, and also added eserine to the soaking solution; this prevented any apparent loss of ACh in subsequent experiments.

From the total amount of ACh which diffused out of these muscles, it is clear that added ACh is not distributed freely throughout the muscle. In five diaphragms which remained attached to the ribs while soaking in ACh the mean ACh space was 29·0 (S.E. $\pm$ 2·4) ml./100 g muscle. In four muscles cut away from the ribs before soaking in ACh the mean ACh space was substantially greater, 37·7 $\pm$ 0·8 ml./100 g.

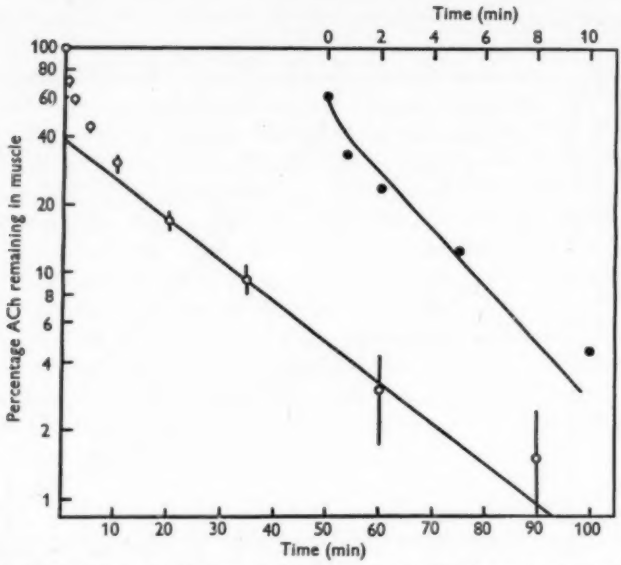


Fig. 3. Rate of loss of ACh from rat diaphragms cut away from rib margin before prolonged soaking in 5×10^{-4} M ACh. Each point (○) is mean of 4 observations with $\pm 1 \times$ S.E. Fast initial component (●), obtained by subtraction, is shown on expanded time scale, with diffusion curve calculated by using $D/l^2 = 0{\cdot}002$. Semi-log. scale.

DISCUSSION

Diffusion of ACh in agar gels

There is good reason to believe that dilute agar gels do not slow down diffusion appreciably. Stiles & Adair (1921), studying the penetration of NaCl into 0·5 % agar gels, found no evidence of slowing caused by the agar particles. In fact, diffusion seemed to be slightly faster than the reported diffusion of NaCl in pure water; but, as they pointed out, this difference was probably illusory, arising from uncertainty about the real concentration of Na and Cl ions in the region of diffusion in the two cases.

The relatively high value for D which we have obtained ($9{\cdot}8 \times 10^{-6}$ cm^2/sec) provides further reason for believing that diffusion was not appreciably slower in our experiments than it would have been in the absence of agar. Fatt (1954) has calculated a value for D of 8×10^{-6} cm^2/sec from an estimate of the limiting equivalent conductance of ACh^+ at 18° C. At the ionic strength of Ringer–Locke solution one would expect this figure to be reduced by approximately 10 %. There seems to be no obvious explanation why our figure is somewhat higher than this. It is unlikely that Cl^- could

much accelerate the movement of ACh^+ in the presence of relatively high concentrations of other ions, which would prevent the generation of any appreciable diffusion potentials.

The temperature coefficient of D between 20 and 36·5° C was about 2 % per degree, which is of the usual order of magnitude.

A slightly puzzling feature of the diffusion experiments at room temperature was that the straight line which could regularly be drawn through graphs of Q against $\sqrt{t}$ rather consistently tended to cross the abscissae at some positive value of t. Reference to Equation (2), which describes the combined results, shows that at zero time Q was not zero but $-3{\cdot}54$ (with a S.E. of $\pm 1{\cdot}31$); the probability of obtaining such a large deviation from zero by chance is only 0·01 (experiments at $36{\cdot}5 \pm 1{\cdot}5°$ C did not show a significant initial delay of diffusion; the value of Q for zero time was $0{\cdot}95 \pm 1{\cdot}11$).

It is of some interest that a similar delay at the beginning of diffusion was observed by Stiles (1920), whilst studying the penetration of NaCl into an agar gel. It seems that the surface of a gel exposed to the air has a somewhat reduced permeability, which slows initial diffusion when a solution is poured on to the gel.

Outward diffusion of ACh from the diaphragm

Not all the ACh which entered the muscle diffused out freely. A small fraction (some 10–20 % of the total) only left the muscle with a half-time of about 30 min (Fig. 2). The remainder, however, moved out at a rate consistent with free diffusion in a system 50 % thicker than the diaphragms (1·4:0·94 mm). This would be in agreement with the possibility that ACh in the main does not enter the muscle fibres and that the fast component of outward movement represents extracellular diffusion slowed by closely packed impermeable cylinders. Theoretical considerations would lead one to expect the diffusion path to be lengthened by $\frac{1}{2}\pi$. Such good agreement with expectation is likely to be fortuitous; previous authors have found the apparent extracellular diffusion path in muscle of ions such as Na^+ increased by substantially more than $\frac{1}{2}\pi$ (Harris & Burn, 1949; Keynes, 1954; Creese, 1954), probably because other factors also interfere with free diffusion of ions in muscle. The rate of diffusion of ACh during the rapid phase of outward movement was 1·7 and 3·0 times faster than the outward diffusion of ^{131}I-labelled decamethonium and curare, respectively, observed by Creese, Taylor & Tilton (1959) in the rat diaphragm at 38° C.

It can be concluded that at least 4/5 of the ACh added to a diaphragm diffuses out quickly, but it may, nevertheless, take some 7 min for the extracellular ACh to be reduced to 1/10 of the initial amount. This may be of some significance for the function of muscle, particularly when the de-

struction of ACh liberated during activity is prevented by saturation of cholinesterases or through the action of drugs (cf. the eserine 'slow wave' of Eccles, Katz & Kuffler, 1942). Furthermore, even if ACh is destroyed by hydrolysis, there will be a temporary accumulation of choline which may have some physiological action (Hutter, 1952; del Castillo & Katz, 1957).

Diffusion of ACh through the diaphragm

The very low diffusion coefficient of ACh calculated from its movement through the diaphragm ($1{\cdot}4 \times 10^{-6}$ cm^2/sec) also suggests that ACh was unable to diffuse freely through the muscle fibres. If we assume that the diffusion path through the full thickness of the diaphragm was increased by 50 %, the apparent value of D becomes $2{\cdot}1 \times 10^{-6}$ cm^2/sec. It would then follow that the area available for diffusion was only about 0·21 of the total area.

Distribution of ACh in the diaphragm

Our results show that the ACh recoverable from muscles not separated from their ribs, after prolonged soaking, corresponds to a total ACh space of 29 ml./100 g of intact muscle. It is difficult to separate the diaphragm from its insertion into the costal margin without cutting the muscle fibres; this procedure usually causes the fibres to twitch and, when followed by soaking in ACh, results in a higher value for the ACh space, 37·7 ml./100 g (cf. also the very different estimates of the inulin space in the diaphragm made by Creese, 1954, and Pappius & Elliott, 1956). Extrapolating the slow phase of outward movement to zero time has shown that about 85 % of the ACh is free to diffuse out from intact muscles. The extracellular or 'free' ACh space in the diaphragm is therefore $0{\cdot}85 \times 29$ or about 25 ml./100 g of muscle, in excellent agreement with the mean value of 26·4 ml./100 g obtained by Creese (1954) for the inulin space in the isolated diaphragm. It may be objected that it is not justifiable to extrapolate in this way to zero time, because the rate of movement from the slow component should initially be much reduced by the high extracellular level of ACh (cf. Keynes, 1954, and Dainty & Krnjević, 1955). Inspection of the results suggests that a value of about 12 % (instead of 15 %) might be a better approximation to the true slow fraction of ACh, but the scatter of data hardly justifies a more detailed analysis. In any case the exact value of the slow fraction is not at all critical; it could be anywhere between 0 and 25 % without materially affecting the conclusion that the free ACh space agrees quite well with the inulin space.

The belief that ACh remains mostly outside the cells is thus supported both by the slowness of its diffusion through the diaphragm, and its apparent distribution within it. This is, of course, not wholly unexpected; direct measurements of the entry of labelled ACh into the squid giant axon

have shown only extremely slow penetration (Rothenberg, Sprinson & Nachmansohn, 1948), and the demonstration by del Castillo & Katz (1955) that ACh depolarizes the end-plate membrane only if applied to the outside, and not the inside, of the muscle fibre is not consistent with a high degree of permeability.

'Slow' ACh

Few definite statements can be made about the nature of the slow component of ACh diffusion in the diaphragm, except that it is extremely unlikely to be ACh released spontaneously by the nerve endings. In the diffusion experiments over a period of 1–2 hr the slow component corresponded to 50–70 nmole of ACh; spontaneous release would not be expected to amount to much more than 0·1 nmole in that time (Krnjević & Mitchell, 1960), and even maximal activation of the phrenic nerve would release less than 2 nmole in 2 hr (Straughan, 1960). It seems probable that the slow component represents some penetration of the muscle fibres, but we have not excluded the possibility that it may be caused at least partly by binding of ACh to some extracellular tissue component, or even to the surface of the fibres, perhaps at the receptor sites.

SUMMARY

1. The rate of movement of ACh from a Ringer–Locke-agar gel into well-stirred Ringer–Locke solution corresponds to a diffusion coefficient of 9·8 (S.E. $\pm$ 0·42) $\times 10^{-6}$ cm^2/sec at 20 $\pm$ 1° C.

2. Under steady-state conditions the diffusion of ACh across the rat diaphragm, from a solution containing a high concentration to one containing virtually none, occurs at a rate which is only 1/7 of that expected from diffusion in agar gels.

3. After soaking the rat diaphragm in solutions of ACh (5×10^{-4} M) for 1–2 hr, 80–90 % of the ACh in the muscle ('free ACh') escapes at the rate expected from simple diffusion; the remaining 10–20 % leaves much more slowly with a half-time of about 30 min.

4. The total ACh space in the soaked muscles is 29 (S.E. $\pm$ 2·4) ml./100 g; the 'free ACh' space is about 25 ml./100 g and agrees well with previous estimates of the inulin space.

5. It is concluded that all but a small proportion of the added ACh remains in the extracellular space of the muscle; the rest may enter the fibres or may be 'bound' to some component of the tissue.

REFERENCES

CRANK, J. (1956). *Mathematics of Diffusion*, p. 347. Oxford: Clarendon Press.

CREESE, R. (1954). Measurement of cation fluxes in rat diaphragm. *Proc. Roy. Soc.* B, **142**, 497–513.

CREESE, R., TAYLOR, D. B. & TILTON, B. (1959). The effect of curare on the uptake and release of a depolarizing agent labelled with ^{131}I; in *Curare and Curare-like Agents*, ed. BOVET, D., BOVET-NITTI, F. & MARINI-BETTOLO, G. B., pp. 386–390. Amsterdam: Elsevier.

DAINTY, J. & KRNJEVIĆ, K. (1955). The rates of exchange of ^{24}Na in cat nerves. *J. Physiol.* **128**, 489–503.

DEL CASTILLO, J. & KATZ, B. (1955). On the localization of acetylcholine receptors. *J. Physiol.* **128**, 157–181.

DEL CASTILLO, J. & KATZ, B. (1957). Interaction at end-plate receptors between different choline derivatives. *Proc. Roy. Soc.* B, **146**, 369–381.

ECCLES, J. C., KATZ, B. & KUFFLER, S. W. (1942). Effect of eserine on neuromuscular transmission. *J. Neurophysiol.* **5**, 211–230.

EGGLETON, G. P., EGGLETON, P. & HILL, A. V. (1928). The coefficient of diffusion of lactic acid through muscle. *Proc. Roy. Soc.* B, **103**, 620–628.

FATT, P. (1954). Biophysics of junctional transmission. *Physiol. Rev.* **34**, 674–710.

HARRIS, E. J. & BURN, G. P. (1949). The transfer of sodium and potassium ions between muscle and the surrounding medium. *Trans. Faraday Soc.* **45**, 508–528.

HILL, A. V. (1928). The diffusion of oxygen and lactic acid through tissues. *Proc. Roy. Soc.* B, **104**, 39–96.

HUTTER, O. F. (1952). Effect of choline on neuromuscular transmission in the cat. *J. Physiol.* **117**, 241–250.

KEYNES, R. D. (1954). The ionic fluxes in frog muscle. *Proc. Roy. Soc.* B, **142**, 359–382.

KRNJEVIĆ, K. & MITCHELL, J. F. (1960). The release of acetylcholine in the isolated rat diaphragm. *J. Physiol.* (in the Press).

PAPPIUS, H. M. & ELLIOTT, K. A. C. (1956). Water distribution in incubated slices of brain and other tissues. *Canad. J. Biochem. Physiol.* **34**, 1007–1022.

ROTHENBERG, M. A., SPRINSON, D. B. & NACHMANSOHN, D. (1948). Site of action of acetylcholine. *J. Neurophysiol.* **11**, 111–116.

STEFAN, J. (1871). Über die Diffusion der Kohlensäure durch Wasser und Alkohol. *S.B. Akad. Wiss. Wien*, **77**, Abt. 2, 371–409.

STILES, W. (1920). The penetration of electrolytes into gels. 1. The penetration of sodium chloride into gels of agar-agar containing silver nitrate. *Biochem. J.* **14**, 58–72.

STILES, W. & ADAIR, G. S. (1921). The penetration of electrolytes into gels. 3. The influence of the concentration of the gel on the coefficient of diffusion of sodium chloride. *Biochem. J.* **15**, 620–628.

STRAUGHAN, D. W. (1960). The release of acetylcholine from mammalian motor nerve endings. *Brit. J. Pharmacol.* (in the Press).

J. Physiol. (1960), **153**, *pp.* 573–582
With 4 text-figures
Printed in Great Britain

MECHANICALLY EXCITABLE RECEPTOR UNITS IN THE MANTLE OF THE OCTOPUS AND THEIR CONNEXIONS

BY J. A. B. GRAY*

From the Department of Zoology, University of California, Los Angeles, United States of America

(*Received* 25 *May* 1960)

The opportunity of a short visit to Los Angeles was taken to investigate the central connexions of mechanically sensitive receptor units in the mantle of *Octopus*. This preparation offers an example of the organization of receptor units, which is suitable for study because there are many similar stellar nerves converging on each stellate ganglion and the ganglion is connected to the rest of the central nervous system by a long pallial nerve or mantle connective. The anatomy of this system in *Octopus vulgaris* has been described by Sereni & Young (1932). Figure 1, kindly prepared by Professor J. Z. Young, summarizes those connexions of primary receptor neurones for which there is evidence. The receptor neurones include those having mechanically sensitive receptors in the skin and in the muscle of the mantle wall; evidence for the existence of such receptors and some of their properties are described in the first section of the results. The axons of these neurones, which have their cell bodies in the periphery, run to the brain (Sereni & Young, 1932). There is evidence that mechanically sensitive receptor neurones from both skin and muscle give off branches in the stellate ganglia and that these branches make synaptic contact in the ganglia, either directly or indirectly, with motor neurones. These motor neurones have their cell bodies in the stellate ganglia and send their axons to the muscles of the mantle but not to the chromatophore muscles (Sereni & Young, 1932). The existence of reflex connexions in these ganglia has been discussed and disputed in the past and is considered in the discussion; results supporting the general concept and describing some of the properties of connexions between mechanically sensitive receptor neurones and motor neurones are given in the second section of the results. The motor neurones are also connected with neurones descending from the brain: the behaviour of some of these descending neurones and the way their activity is influenced by activity in receptor neurones are considered in the third section of the results. The final section of results

* Present address: Department of Physiology, University College London.

describes some experiments designed to test a possible explanation of the function of the observed central connexions of mechanically sensitive receptor units.

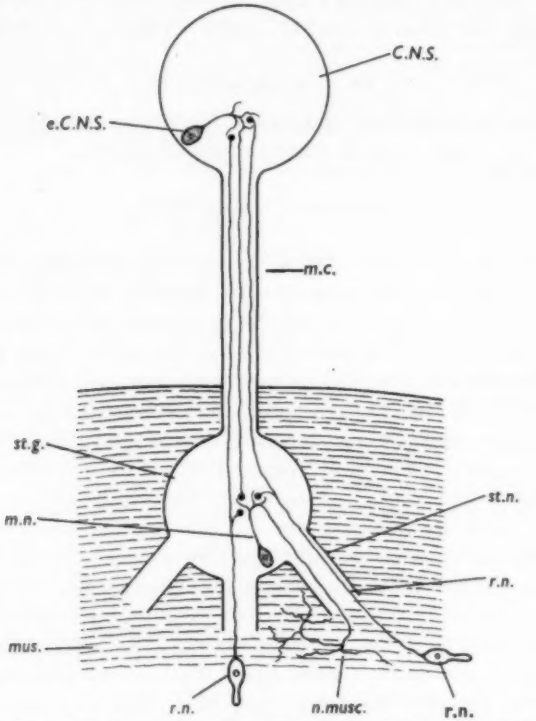


Fig. 1. Diagram of the innervation of the mantle of an Octopod. *C.N.S.*, circumoesophageal ganglia; *e.C.N.S.*, efferent from *C.N.S.*; *m.c.*, mantle connective; *m.n.*, motor neurone; *mus.*, mantle muscles; *n.musc.*, nerves to mantle muscles; *r.n.*, receptor neurone; *st.g.*, stellate ganglion; *st.n.*, stellar nerve. Presynaptic terminals are indicated by small filled circles. The cell bodies of neurones on the motor side are shaded; the cell bodies of the receptor neurones are unshaded (by courtesy of Professor J. Z. Young).

METHODS

The experiments were performed on one or both of two species of *Octopus*, which are common on the south Californian coast, *Octopus bimaculatus* and *Octopus bimaculoides*. The species are not easy to distinguish and no attempt to do so was made. The results showed no inconsistencies which might be attributed to a species difference.

Three types of preparation were used. One was a preparation of mantle, stellate ganglion and connective; this preparation was isolated from the head and visceral mass after killing the animal by cutting into the brain. A second was an anaesthetized preparation; anaesthesia was induced in a 1–2% solution of urethane in sea water and maintained, so that the

animal was quiescent and breathing regularly, with a 0·5% solution. The third type of preparation was one in which the optic and supra-oesophageal lobes of the brain were removed under urethane anaesthesia and the anaesthetic then allowed to pass off. It must be remembered that in a gill-breathing animal urethane is exchanged through the gill membranes and the depth of anaesthesia can be controlled by changing the concentration in the sea water. Even after several hours of anaesthesia an animal transferred to fresh sea water will revive after 5–10 min.

Nerve impulses were recorded with an amplifying and oscilloscope system which had an input impedance of 10 MΩ and a frequency response limited to give the best signal-to-noise ratio for indicating the presence or absence of impulses. The electrodes used were either stainless-steel forceps or entomological pins. No special equipment was prepared for mechanical stimulation; the mantle was touched or pressed with a rod, point or bristle; in some experiments it was stretched with forceps and in others a weight was placed on it. Electrical stimuli were brief pulses provided from a low impedance source.

RESULTS

Receptor units in the mantle. Mechanical stimulation of the mantle caused a discharge of impulses in the peripheral stumps of cut stellar nerves. After removing the skin from the outside and the lining of the mantle cavity from the inside of the muscles, activity could still be obtained in these nerves in response to mechanical stimulation, though the number of active units was less than when the skin was present. Most of the subcutaneous connective tissue was removed with the skin, but except for a few preparations some remained on the outside of the muscle; the mantle cavity surface of the muscle was usually stripped bare. When all the subcutaneous connective tissue had been removed some activity was still seen; but stronger stimuli were required to initiate it.

In some experiments the stellar nerves were divided until single receptor units could be identified. When both skin and muscle were present the majority of units were found to adapt rapidly to a maintained force provided by a weight. These units all had receptive fields about 0·5–1·0 cm across. Some units, after an initial decline in frequency, maintained a discharge of impulses throughout the period of test (Fig. 2). It is unlikely that these maintained discharges were due to vibration of the load, since tapping the table, while the preparation was loaded, had no effect on the observed activity.

The responses of the single units obtained from preparations from which the skin had been stripped were all phasic in type. No maintained discharges were obtained either in response to a steady pressure or to a stretch. This failure to find tonic units cannot be taken as evidence that they do not exist, partly because the sample studied was small and partly because removing the skin must be presumed to cause some damage to the receptor units or to tissue near them. The receptive fields of the units

found in these preparations were somewhat larger than those found when the skin was present, being about 1 cm across.

Connexions of receptor units in the stellate ganglion. A discharge of impulses in the central stump of a cut stellar nerve could be initiated by mechanical stimulation of an area of mantle both before and after stripping away the skin. These responses could only be obtained with ease when the mechanical stimulation was applied in a localized area, perhaps 1–2 cm diameter, in that region of the mantle which would have been supplied by the nerve from which the recording was being made. Even without quantitative means of mechanical stimulation it was clear that the force

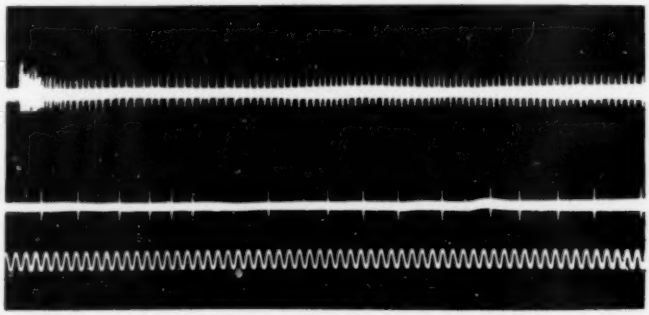


Fig. 2. Impulse discharge in a tonic receptor unit of the mantle (skin and muscle present). The skin side was loaded with 9·7 g at the beginning of the discharge in *a*. *b*, still loaded after an interval of 27 sec. *c*, time 0·1 sec. There was no activity before loading.

required to set up a discharge in the central end of a cut stellar nerve was considerably greater than that required to initiate a discharge in a peripheral stump. This suggested that the discharge in the central stump was a reflex discharge in motor fibres and not simply a centrifugal discharge in another branch of the same receptor neurone; it also suggested that there was some convergence on the motor neurones. The first conclusion is supported by the fact that the muscle could be seen to contract when the mantle, isolated from the brain, was stimulated mechanically. Furthermore, mechanical stimulation of the mantle could facilitate the response seen in the central stump of a stellar nerve after electrical stimulation of the connective (pallial nerve) (Fig. 3). In this experiment a brief electrical shock was applied to the connective once every 2 sec and the stimulus strength adjusted till the response was just visible (Fig. 3*b*). Touching the mantle in the correct area immediately before the shock regularly caused a large increase in the area of the record of the response and a reduction in the latency (Fig. 3*c*); in the experiment illustrated in

Fig. 3 this was 3 msec. The response to the mechanical stimulus *per se* can be seen in the increase in base-line activity before and after the electrically excited response. Increasing the frequency of the electrical stimulation of the connective also caused a large increase in the area of the record of the response, thus confirming the observations of Bullock (1952) and Wilson (1960) on the octopus (*Octopus bimaculatus* and *bimaculoides*). These results indicate that there must be connexions between primary receptor

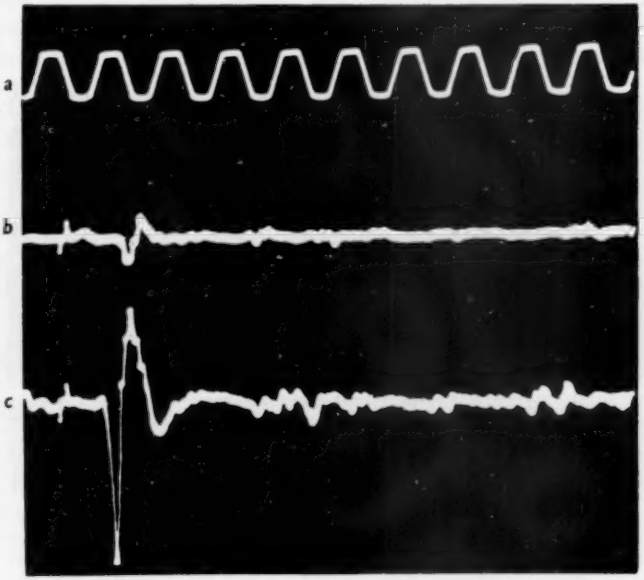


Fig. 3. Facilitation by mechanical stimulation of the mantle of the post-ganglionic response to electrical stimulation of the mantle connective: *a*, time 10 msec; *b*, response to electrical stimulation of the connective alone; *c*, response to the same stimulus during mechanical stimulation of the mantle, the effect of the mechanical stimulus alone can be seen before and after the electrical stimulus.

neurones and the motor neurones of the stellate ganglion; also that there is convergence which may be either between primary receptor neurones, or between these neurones and others which descend from the brain, or, perhaps most likely, both. The experiment just described cannot, however, justify the conclusion that there is convergence with descending fibres, because the electrical stimulus may have been activating receptor fibres, which run up the connective after giving off branches that form synapses with the motor neurones of the ganglion.

Experiments were performed to see if there were connexions with descending neurones. The activity of single motor units was identified by

stripping down the central stump of a stellar nerve in preparations in which the ganglion was left connected to the brain and from which the optic and supra-oesophageal lobes had been removed. Units in the stellar nerves were found that responded rhythmically either with each inspiration or with each expiration. On two occasions a unit, which had fired in relation to breathing until the connective was cut, appeared to be excited by mechanical stimulation of the mantle after the connective had been cut. The words 'appeared to be' have been used in the last sentence since the records were not obtained from anatomically isolated single fibres and the spikes could not be identified with certainty as arising from the same

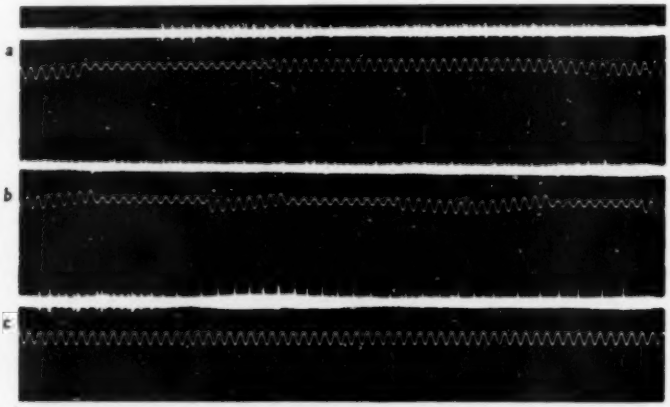


Fig. 4. Activity in units of the mantle connective connected centrally and cut below the electrodes. *a*, normal breathing, one typical cycle, inspiration marked by reduced amplitude of time trace (0·1 sec); *b*, cessation of activity during touching (at each reduction of signal amplitude); *c*, funnel clamped—note prolonged expiration. Phases of respiration not marked.

unit. It is probable that branches from primary receptor neurones converge in the ganglion together with fibres descending from the brain and facilitate the activity of those motor neurones which supply muscles in the region of the receptors. No inhibition was ever seen, but its existence cannot be excluded.

Connexions of receptor units with respiratory and other neurones in the brain. Records were obtained from single units which had been dissected from the central stumps of cut connectives. Some units fired during inspiration and some during expiration. Figure 4*a* is a record in which two units stood out, one firing in each of the phases of breathing. In no experiment was a unit seen to fire during both phases.

Mechanical stimulation of the mantle influenced this activity in a number of ways. The respiratory rhythm might cease altogether (Fig. 4*b*), or it

might increase in frequency and in the intensity of each burst. A common response to mechanical stimulation of the mantle, however, was a discharge of nerve impulses in the connective that was unrelated to the respiratory rhythm. It seemed that some at least of these units did not take part in the rhythmical activity, but further experiments would be required to prove that these groups of neurones are completely dissociated.

Mechanical stimulation of the mantle was found to influence the activity of those neurones firing in phase with respiration but such activity was not necessary to the development of the rhythm. Rhythmical activity was recorded from the central stump of the connective when it was connected only to the posterior half of the suboesophageal lobes, the remainder of the brain had been destroyed and these lobes, together with the stump of one connective, had been isolated from the rest of the animal except for the surrounding cartilage. The frequency of the rhythm was little changed either by cutting the nerves which carry the receptor fibres from the mantle and the visceral mass, or by isolation of part of the brain as just described (cf. Mislin, 1955).

Clamping the funnel prevents the discharge of water from the mantle cavity and this procedure increased the duration of the impulse discharge of expiratory units (Fig. 4*c*); this procedure was only tried when the peripheral nerves were connected to the brain.

Physiological function of mechanically sensitive receptors of the mantle. Three experiments were performed in which stellar nerves were found through small openings in the outside of the mantle. The peripheral stumps of these nerves could be prepared for recording without breaking the continuity of the lining of the mantle cavity. The animals used were either anaesthetized or had had their optic and supra-oesophageal lobes removed. Recordings were made from whole peripheral stumps, which responded to mechanical stimulation of the skin with a vigorous discharge of impulses. No impulses were ever seen during normal breathing. Clamping the funnel caused a prolonged expiratory effort, but even under these conditions no impulses were ever seen in receptor units from the mantle. It was thought that, if stretching the mantle were a physiological stimulus and if receptor activity controlled the uniformity of the mantle contraction, then activity in receptor units should be at a maximum in a part of the mantle that was not itself contracting. In order to achieve this situation, the connective was cut on the side from which the recording was being made. No impulses were seen in any receptor unit either during normal breathing movements or during forced expiration induced by clamping the funnel.

DISCUSSION

Mechanically sensitive receptor units have been found, not only in the skin, but also in other parts of the mantle. The receptors responsible for the activity found in skin preparations may be associated with the cells described by Mikhailoff (1921) and Sereni & Young (1932) in the outermost layers of the muscle; in such a position the receptors would be liable to damage during the removal of the innermost layers of the subcutaneous connective tissues, and this could explain the reduction of activity found when these layers were removed. The presence, in the stellate ganglia, of connexions which allow mechanically excited receptor units to facilitate or even activate neighbouring motor neurones suggests that there might be a feedback mechanism for ensuring that the contraction of the mantle muscle was always uniform. However, the absence of activity in any receptor units in the mantle during breathing movements under any of the conditions tried would seem to exclude this hypothesis: Young (1939) has shown that there are mechanisms of a different type that ensure uniform contraction in the squid mantle.

There has been a long controversy as to whether or not there are reflex connexions in the stellate ganglion. Fröhlich (1910) and Ten Cate (1929) using *Octopus vulgaris* both produced evidence in favour of a reflex connexion. Others (see Sereni & Young, 1932) came to the opposite conclusion. Sereni & Young (1932) thought that an axon reflex had not been excluded. Recent work by Wilson (1960) supports the reflex hypothesis. The evidence presented in this paper, particularly the evidence that the post-ganglionic response to stimulation of the mantle connective can be greatly facilitated by mechanical stimulation of the mantle, confirms the view that mechanically sensitive receptor units make synaptic connexions in the stellate ganglion.

The connexions between receptor units and motor neurones in the ganglion are such as to give a powerful local facilitation. If the receptor units running in the nerve from which the recordings were made could have been left uncut, it is probable that the effectiveness of the facilitation would have proved greater still. When the hypothesis of stretch reflexes already discussed is discarded it is difficult to understand the physiological significance of this localized connexion. Touching the mantle of a normal unanaesthetized animal does not appear to produce any irregularity in the contraction of the mantle; usually no response is observed until the animal moves away, and it is clear that factors other than the mechanical stimulation of the mantle skin play a part in this over-all response. If the animal swims away, using its mantle for the purpose, the contractions are apparently symmetrical, as one would expect: it would, however, require

high-speed photography to be certain about this. The reflex may be associated with the animal's habit of living in rock crannies, but there is no evidence to support this suggestion.

The finding that there are separate units in the connective that are active in inspiration and in expiration might be expected after the findings of von Uexküll (1895) on *Eledone* and Boycott (1960) on *Sepia* that inspiration and expiration can be excited separately by stimulation of the brain. It is interesting that separate channels are used between the brain and the ganglion to initiate the two phases of breathing rather than a single channel with a frequency code; the latter would be economical in space but would be less reliable and reliability in such an important function must be of great importance for survival. The rhythm of breathing is determined in the brain and is not significantly affected by cutting off the inflow from receptors; this is not surprising in view of the failure to detect any receptor activity during breathing movements.

The organization of the mechanically sensitive receptors of the mantle in relation to the motor control of the mantle muscle is a problem that could be worked out in detail. Even though the biological significance of these connexions is still obscure, such an investigation might lead to a greater understanding of the general principles underlying the peripheral organization of receptor nervous systems.

SUMMARY

1. Phasic receptor units responding to mechanical stimulation of the skin and of the muscle of the mantle of *Octopus* have been observed. Tonic units were found in the skin.

2. Units from skin and muscle have reflex connexions in the stellate ganglia and converge with other fibres on to motor neurones. Reflex responses are concentrated in the region adjacent to the point of stimulation.

3. Units active in inspiration and units active in expiration are found in the mantle connectives (pallial nerves). The rhythm is little affected by cutting the mantle and visceral connectives or by isolation of the posterior parts of the infra-oesophageal lobes. The activity of these units and probably of others is affected in a number of ways by mechanical stimulation of the mantle.

4. Receptor units in the mantle do not fire during quiet breathing or under experimental conditions designed to increase the stretch of the receptors.

5. The system investigated does not appear to control the uniformity of the contraction of the mantle, and its functional significance is still unknown.

My thanks are due to Dr T. H. Bullock for his help, to the Wellcome Trust for a travel grant and to the National Institute for Neurological Diseases and Blindness for support (grant B21).

REFERENCES

BOYCOTT, B. B. (1960). The functional organization of the brain of the cuttlefish, *Sepia officialis* L. *Phil. Trans.* (in the Press).

BULLOCK, T. H. (1952). The invertebrate neuron junction. *Cold Spr. Harb. Symp. quant. Biol.* **17**, 267–273.

FRÖHLICH, F. W. (1910). Experimentelle Studien am Nervensystem der Mollusken. (1) Das Mantelganglion der Cephalopoden als Reflexorgan. *Z. allg. Physiol.* **10**, 384–390.

MIKHAILOFF, S. (1921). Système nerveux cellulaire périphérique des Céphalopodes. *Bull. Inst. Oceanogr. Monaco, N.* 402.

MISLIN, H. (1955). Die rhythmischen Spontanentladungen im Zentralnervensystem der Tintenfische. *Acta med. scand.*, Suppl. **307**, **152**, 58.

SERENI, E. & YOUNG, J. Z. (1932). Nervous degeneration and regeneration in *cephalopods*. *Publ. Staz. Zool. Napoli*, **12**, 173–208.

TEN CATE, J. (1929). Sur la question de l'excitabilité réflexe du ganglion stellaire. *Arch. Neurol. Physiol.* **14**, 1–6.

VON UEXKÜLL, J. (1895). Physiologische Untersuchungen an Eledone moschata. IV. Zur Analyse der Functionen des Centralnervensystems. *Z. Biol.* **31**, 584–609.

WILSON, D. (1960). Nervous control of movement in cephalopods. *J. exp. Biol.* **37**, 57–72.

YOUNG, J. Z. (1939). Fused neurons and synaptic contacts in the giant nerve fibres of cephalopods. *Phil. Trans.* B, **229**, 465–503.

J. Physiol. (1960), **153**, *pp.* 583–594
With 4 text-figures
Printed in Great Britain

RECEPTIVE FIELDS OF GANGLION CELLS IN THE CAT'S RETINA

BY T. N. WIESEL*

Neurophysiological Laboratory, Department of Pharmacology, Harvard Medical School, Boston 15, *Massachusetts, U.S.A.*

(*Received* 10 *June* 1960)

In his analysis of retinal ganglion cells in different mammals, including the cat, Granit (1947, 1955) found that diffuse illumination of the retina gave rise to three types of responses: depending on the type of discharge the different ganglion cells were called 'on' units, 'off' units and 'on-off' units. The discharges were similar to those previously found by Hartline (1938) in the frog. Using small-spot stimulation, Kuffler (1953) has shown that on the basis of their receptive field organization there are only two types of ganglion cells in the cat retina. One gives 'on' discharges when the centre is stimulated and 'off' discharges to illumination of the periphery of the receptive field. The other type shows the reverse arrangement with 'off' responses in the centre and 'on' responses in the surrounding area. The 'on-off' response to diffuse illumination is the result of a combined contribution from the centre and periphery of receptive fields. Kuffler also showed that the central and peripheral portions within each receptive field are mutually antagonistic. These findings in the cat have subsequently been extended and confirmed (Barlow, FitzHugh & Kuffler, 1957*a*, *b*; Wiesel, 1958; Hubel, 1960).

The main object of the present study of retinal ganglion cells was to examine the size of field centres and the modification of discharges by inclusion of the surrounding area. The method of Barlow (1953) using light spots of different sizes and measuring thresholds for influencing the discharge from individual ganglion cells was found most useful. This area–threshold technique revealed striking differences between individual ganglion cells and between cells in the area centralis and the periphery of the retina. A preliminary account of some of this work has been published (Wiesel & Brown, 1958).

METHODS

A detailed description of the method used in these experiments has recently been given (Brown & Wiesel, 1959). Twenty-six cats were kept under light anaesthesia with intraperitoneal injections of sodium pentobarbital (initial dose 20–30 mg/kg) and Dial (allobarbitone; Ciba)

* The work reported here was done at the Wilmer Institute, Johns Hopkins University, School of Medicine, Baltimore, Maryland.

with urethane (initial dose 0·25 ml./kg). The eyes were immobilized by continuous intravenous infusion of succinylcholine (about 10 mg/kg/hr), which made artificial respiration necessary. Atropine in 1 % solution was used to dilate the pupils and relax accommodation. The corneas were kept moist and clear with contact lenses and buffered lens solution.

The multibeam ophthalmoscope of Talbot & Kuffler (1952) was used for stimulation. This provided a steady background retinal illumination of 0·34 log lm/m² over an area 4 mm (16°) in diameter. Stimuli consisted of spots of light of various sizes with a maximum intensity of 1·85 log lm/m². Intensity of illumination was regulated by circular wedges. Spot sizes, centred in the area under background illumination, varied in steps from 0·125 mm (0·5°) to 3 mm (12°) in diameter. The spots and background could be positioned in different regions of the retina.

Electrical recordings from the retina of the intact eye were made with micro-electrodes, guided through the sclera by a hypodermic needle, approaching the retina from the vitreous side. A ball-joint arrangement made it possible to direct the electrode to different parts of the retina (Talbot & Kuffler, 1952). The electrode was advanced by a hydraulic system. Two types of electrode were employed. The most commonly used were 3 M-KCl-filled glass micropipettes with tip diameters less than 0·5 μ and d.c. resistances of 15–30 MΩ. The other type was a 10–15 μ glass-insulated platinum electrode similar to that developed by Granit & Svaetichin (1939). A negative-capacitance pre-amplifier with a grid current of 5×10^{-12} A was fed into a wide-band a.c.–d.c. amplifier which was connected to an oscilloscope.

The threshold of a stimulus spot was given by the lowest intensity of light which produced a clear and repeatable change in the maintained impulse activity. The change in discharge frequency was detected by observing the impulses on an oscilloscope and by listening to a loudspeaker. Each stimulus lasted 0·8 sec and was repeated every 10 sec. Several wedge readings were taken, first with increasing and then with decreasing spot sizes. Adaptive effects due to the recurrent stimulus were not observed, presumably because the background illumination was well within photopic range.

RESULTS

Receptive field size

Single-unit activity was recorded from a few hundred ganglion cells in various regions of the cat's retina. Micropipettes recorded this activity within the retina at depths up to 30–40 μ (Brown & Wiesel, 1959), whereas platinum electrodes only recorded from the surface of the retina. Cells were continuously active during steady background illumination; this maintained activity showed great variation from unit to unit (Kuffler, FitzHugh & Barlow, 1957). After a ganglion cell was located its receptive field was first mapped out by the smallest spot that gave clear on- or off-responses. Each receptive field had a centre more or less circular in shape, surrounded by a peripheral zone. The total receptive-field sizes including both centre and periphery were usually 2–3 mm in diameter. On- and off-centre units were recorded in about equal numbers both from the area centralis and from the periphery of the retina.

The size of receptive field centres was determined by the area–threshold method. As the size of a centred spot increased, the threshold for the centre response usually decreased; similarly if the stimulus intensity

was kept constant the centre response itself increased. Thus, there was summation over this area. Increasing the spot beyond the size of the field centre did not cause a further lowering of threshold, but, on the contrary, the threshold actually rose as more of the periphery was included (Barlow *et al.* 1957*b*).

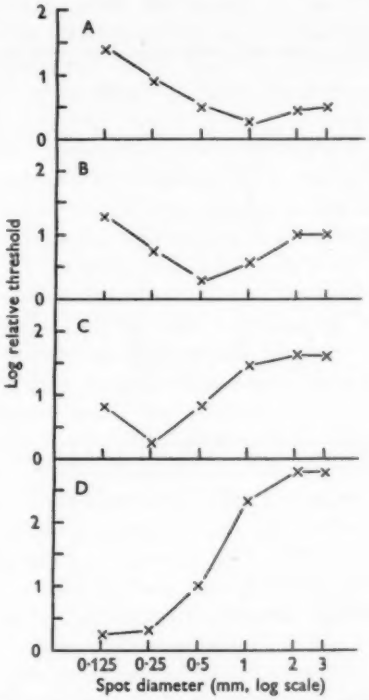
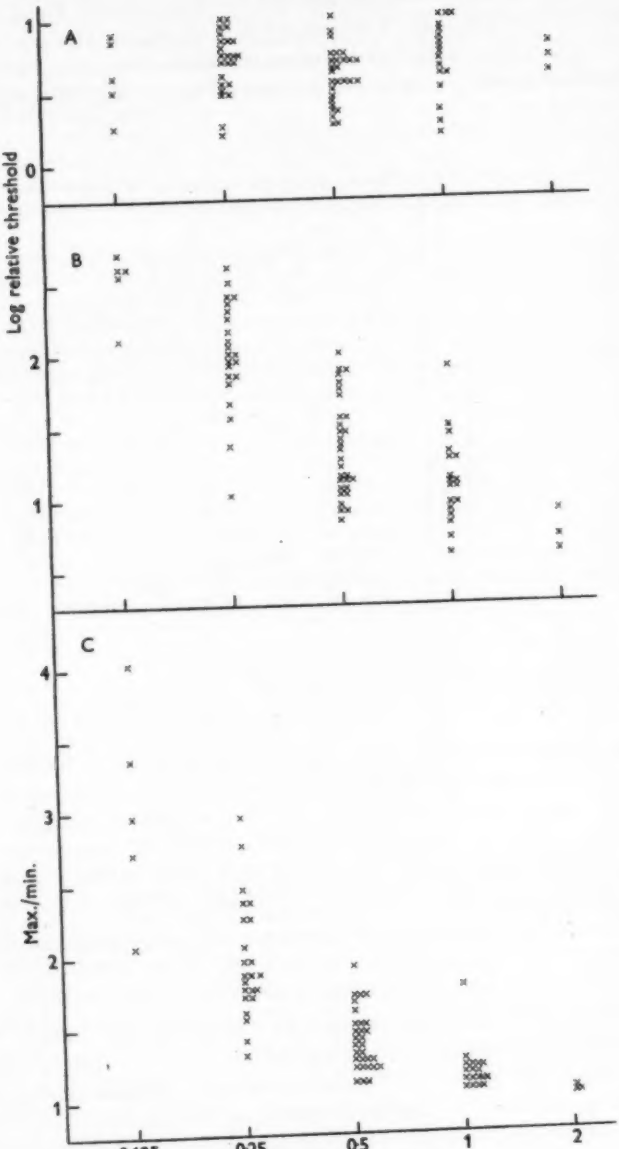


Fig. 1. Changes in threshold of centre responses with area of illumination measured for four different ganglion cells, all 'on'-centre units. Abscissa: spot diameter. Ordinate: threshold, relative logarithmic scale: 0 corresponds to a stimulus of 2·35 log lm/m². Background retinal illumination 0·34 log lm/m². Stimulus duration 0·8 sec.

The relation between stimulus size and response was mainly studied with threshold stimulation. However, similar results were obtained by using supra-threshold stimuli of constant intensity and measuring latency and impulse frequency for different spot sizes (see also FitzHugh, 1957). Because of the effects of scattered light, measurements at more than about 2 log units above threshold intensities were considered unreliable.

Figure 1 gives a representative sample of area–threshold curves from



For legend see foot of opposite page.

which the sizes of receptive field centres can be determined. For cell A there was summation of centre responses up to 1 mm., i.e. its centre area was about 1 mm in diameter. In B the centre response summed over 0·5 mm, while in C the area was 0·25 mm in diameter. The ganglion cell of Fig. 1D had its lowest threshold for the smallest available spot, 0·125 mm in diameter. The optimum spot for ganglion cell A (Fig. 1) was thus sixty-four times that of cell D. Since the spot sizes were varied in fixed, rather coarse, steps, this ratio may not accurately reflect the ratio of the receptive field centre areas. However, it is clear that the centre areas must be strikingly different in size.

The antagonistic influence of the periphery of the receptive field on the centre can also be seen in Fig. 1. There was a rise in threshold of centre response as more of the surround was included in the area of illumination. The amount of this peripheral suppression of centre response varied from one cell to another. In the cell of Fig. 1D the difference in threshold for a spot 0·125 mm in diameter, illuminating only the centre, and a 2 mm spot covering the whole receptive field, was about 2·5 log units. The cells in B and C showed less peripheral suppression, and the cell in A with the largest centre showed only a small difference in threshold between centre and whole-field illumination.

For the units of Fig. 1, when the spots were increased beyond 2 mm in diameter there was no further change in threshold for the centre response. Presumably the spots were now larger than the receptive fields. Sizes of receptive fields measured by the area–threshold method agreed reasonably well with those obtained by mapping fields with small spots. Thus the total field diameter for each of these four ganglion cells was about 2 mm, despite the differences in centre size. Obviously, cells with smaller centres have relatively larger peripheral zones (as seen in Fig. 1) and ganglion cells with larger centres have narrower peripheral zones.

In comparing receptive fields striking differences were thus found in the size of centre portions. Cells were therefore divided into groups according to the size of centre areas and were studied by illumination of (1) only the centre portion and (2) both centre and periphery. In Fig. 2, eighty ganglion

Fig. 2. Eighty ganglion cells divided into five groups depending on sizes of the receptive-field centres. Abscissa: diameter of centre area (optimum spot). Stimulus parameters same as in Fig. 1. A. Distribution of threshold values of centre responses with illumination confined to the centre portion of the receptive field (optimum spot stimulation); ordinate: log. relative threshold. B. Distribution of threshold values of centre responses for the different groups on stimulation of both centre and periphery (whole-field illumination). Stimulus spot 3 mm in diameter. Ordinate: log. relative threshold. C. Ordinate: the ratio, taken for individual ganglion cells, of threshold values at whole-field illumination to threshold values at optimum spot stimulation.

cells are divided into five groups according to the size of the optimum spots; these groups are marked on the abscissa. Threshold intensities of optimum-size spots are shown in A. Threshold values were scattered over about 1 log unit for ganglion cells in the same group. All groups had this scattering and there seemed to be no systematic variations of threshold with size of optimum spot. This would indicate that if illumination is restricted to the centre region of receptive fields the sensitivity is not related to the size of the centre. These measurements were all made in the light-adapted state, well within the photopic range of background illumination: the situation may be quite different in the dark-adapted state.

At whole-field illumination, or diffuse light stimulation, when centre and periphery were stimulated simultaneously, differences in thresholds for centre responses ('on' or 'off', depending on the cell) varied with the size of the centres. In Fig. 2B a spot 3 mm in diameter was used, and threshold values were plotted for the same groups of ganglion cells as in Fig. 2A. It is clear that to obtain a response with such a large spot a strong stimulus was necessary if the centre of the receptive field was small. Ganglion cells with large receptive field centres, on the other hand, were influenced at much weaker intensities; threshold values for whole-field illumination could be as much as 2 log. units lower for large centre compared with small-centre ganglion cells.

The differences in sensitivity between centre-field and whole-field illumination were usually not great for large-centre ganglion cells, indicating weak antagonism from the periphery of the receptive field. The small-centre ganglion cells, however, showed strong peripheral suppression of centre responses. This suppression of centre responses from the periphery of the field is also expressed in Fig. 2C. Here the ratio between threshold values for the spot covering the entire field (B) and for the optimum spot (A) is taken for the ganglion cells in the different groups. The ratio is large when the antagonism from the periphery is large, and approaches unity as the peripheral influence on the centre response becomes insignificant. In other words, ganglion cells with small centre areas have a high ratio and this ratio tends towards unity with increasing centre areas.

Peripheral-type responses

So far only centre responses from ganglion cells have been considered. That is, in determining thresholds attention was focused on the 'on' response for 'on' centre units, and on the 'on'-suppression and 'off' discharge for 'off' centre units. If, for instance, in an 'on' centre unit, an 'off' discharge developed as the periphery was invaded, this part of the response was ignored in determining thresholds. For each ganglion cell, however, responses from the periphery of the receptive field can be tested

in the same manner as for the centre. The periphery was stimulated by annular stimuli, i.e. rings of light which did not illuminate the centre. The outside diameter was kept at 3 mm, centred on the field, and the inside diameter was constricted so as to include more and more of the receptive field. In Fig. 3 threshold values are shown for a ganglion cell stimulated by spots and annuli. The solid line represents the area–threshold curve for the centre response as already described in Fig. 1. The broken line shows the threshold values of the peripheral response for different sized annuli. The inside diameters of the annuli are plotted on the abscissa together with spot diameters, so that for the annulus the area of stimulation increases when reading the curve from right to left. Both types of responses showed summation within their respective regions: the centre response summed within an area of 0·5 mm in diameter, whereas the summation of the peripheral response was over an area given by an annulus of 0·5 mm inside and 3 mm outside diameter. The presence of mutual antagonism can also be deduced from the two curves: the threshold of the centre response increased when the stimulus spot invaded the periphery of the receptive field and similarly the peripheral response had higher threshold when the annulus included part of the centre in the area of illumination.

For annuli of optimum size the threshold values for peripheral responses were always higher than thresholds obtained with optimum spots for centre responses (see Fig. 3); sometimes this difference between centre and peripheral thresholds was very small. Some ganglion cells gave 'on-off' responses to whole field illumination at threshold intensities. This was true for both 'on' centre and 'off' centre units but was somewhat more common for 'off' centre units. Since the thresholds of centre and periphery may be equal or almost so, it is not surprising that 'on-off' responses are often encountered at whole-field illumination (Granit, 1947).

Comparison of ganglion cells in the area centralis and in the periphery of the retina

The area centralis in the cat is homologous with the foveal region of certain other species. It can be recognized by the relative absence of vessels, by the radial arrangement of surrounding vessels and by differences in pigmentation. There are also electrical criteria for finding the area centralis. Thus a platinum electrode touching the peripheral retinal surface records mass discharges from non-myelinated axons, which are just under the inner limiting membrane. These axons are from more peripherally located ganglion cells on their way towards the optic disk. The area centralis is practically free from such 'passing' fibres and consequently no mass discharges of axons are recorded from this region. These different criteria made it possible to position an electrode either within the region

for central vision or else in clearly peripheral parts of the retina. No comparison was made between different parts of the periphery, and only that region of the retina which has a tapetum was studied.

The over-all receptive field diameters measured with the area–threshold method were between 1·5 and 3 mm, but no striking differences in total field size were found between cells in the area centralis and the periphery

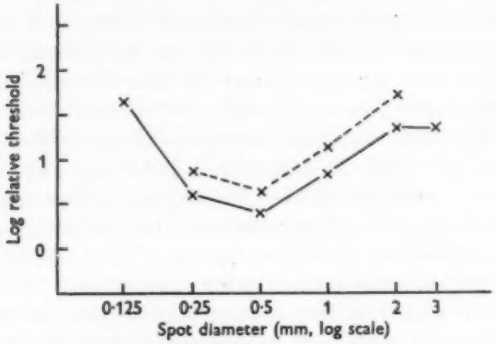


Fig. 3. Area–threshold curves for both centre- and peripheral-type responses of a ganglion cell recorded in the middle periphery about 15° from the area centralis. Abscissa: diameters of spots evoking centre-type responses or inside diameters of annuli producing peripheral-type responses. Outside diameter 3 mm for all annuli. Ordinate: thresholds, relative log. scale. Stimulus conditions as in Fig. 1. ——, area–threshold curve for centre-type response; – – –, area–threshold curve for peripheral-type response.

of the retina. In contrast, the sizes of centre portions of receptive fields showed a difference in distribution between the two retinal regions. In Fig. 4A ganglion cells are again grouped according to the area over which the centre response summed. In each group the white columns represent units from the area centralis and the black columns units with receptive fields in the periphery of the retina. Eighty-two units are included in the histogram: forty-nine of these are from the area centralis, and thirty-three from the periphery. The majority of ganglion cells from the area centralis summed only over an area up to 0·25 mm in diameter, whereas most cells from the periphery showed summation over a larger area.

In the lower histogram (Fig. 4B) are included eighteen cells from the area centralis (white columns) and seventeen cells from the periphery (black columns) all of which were recorded with platinum electrodes. With this electrode, as with the pipette, the centres of cells from the area centralis tended to be smaller than those of cells recorded in the periphery. However, the much larger platinum electrode did not record ganglion cells

with centres smaller than 0·5 mm in diameter; these were quite frequently found with micropipettes.

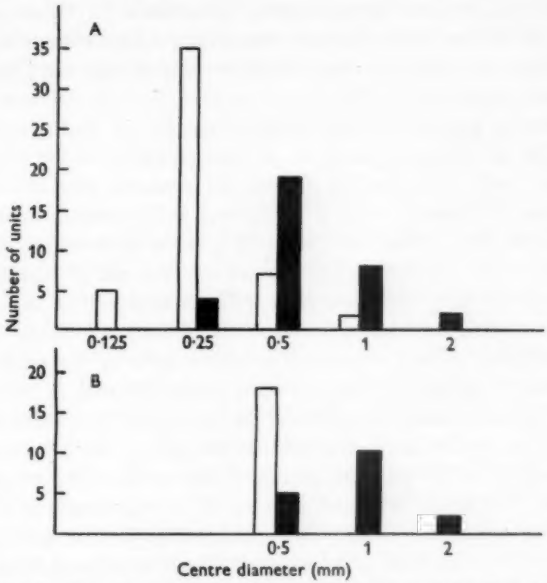


Fig. 4. Distribution of receptive field centres with respect to diameter. White columns indicate cells recorded in the area centralis, black columns, cells from the periphery of the retina. Abscissa: diameter of centre of receptive field. Ordinate: number of cells. A. Eighty-two ganglion cells recorded with micropipette electrodes; forty-nine are from the area centralis and thirty-three from the periphery. B. Thirty-five cells recorded with platinum electrodes; eighteen from the area centralis and seventeen from the periphery of the retina.

DISCUSSION

A comparison of single unit responses in the visual system of the cat indicates that the balance between excitatory and inhibitory influences becomes more critical at successively higher levels (Hubel & Wiesel, 1959). A similar trend was observed in this study for ganglion cells if central and peripheral regions were compared. It was, for example, found that in order to be influenced by diffuse light ganglion cells in the area centralis required much more intense light than peripheral cells. This does not necessarily mean that ganglion cell responses in the area centralis are genuinely less sensitive to any light stimulation, since if only the centre portions of cells in the two regions were illuminated they had similar sensitivity. This is probably related to the fact that ganglion cells in the

area centralis tend to have small but concentrated centre portions and relatively large, strongly antagonistic peripheral zones. Ganglion cells in the periphery, on the other hand, tend to have larger centres and relatively narrow and less influential surrounds. It should be noted that these findings apply to the light-adapted retina only. In darkness the relative influence of the surround is diminished or may disappear (Barlow *et al.* 1957*b*; Wiesel & Brown, 1958).

The relatively large receptive fields, 6° to 12° in diameter, found for ganglion cells in this and previous studies (Kuffler, 1953; Barlow *et al.* 1957*b*) would seem at first sight difficult to reconcile with the high visual acuity of the cat (Smith, 1936). However, the important dimension of receptive fields from the standpoint of acuity is probably the size of the centre portion. In these experiments the smallest available spot had a diameter of 30 min; ganglion cells with smaller centre regions, which may be abundant, could not be studied. Receptive fields with small centres have been found in retinal ganglion cells in the spider monkey by a projection method of retinal stimulation (Hubel & Wiesel, 1960). No recordings were made from foveal ganglion cells but units with receptive fields close to the fovea had centre diameters down to 4 min of arc. Further away from the macula the centre diameters became progressively larger. Thus the receptive field centres of foveal ganglion cells in the monkey may approach in size the minimum separable acuity of about 1 min of arc, determined for primates with a well developed fovea (Grether, 1941). In a discussion of possible mechanisms in high-acuity vision not only the centre size should be considered; the antagonistic peripheral portion of receptive fields is probably equally important. Peripheral suppression of the centre response renders big spots relatively ineffective; centre-size spots thus become the most powerful stimuli. Furthermore, a sharp border between centre and periphery of receptive fields is very likely significant for good visual performance, particularly in respect to moving images, where movement from an 'off' to an 'on' region is doubtless a powerful stimulus (Hubel & Wiesel, 1959).

In recordings from retinal ganglion cells in the cat Rushton (1949) concluded that a 25 μ platinum electrode recorded mainly from very large, 'giant' ganglion cells. He felt that ganglion cells recorded by this method may not be representative for the population of ganglion cells, in which small cells predominate. Kuffler (1953) also found that platinum electrodes (10–15 μ) selected larger ganglion cells, which, however, could be smaller than the 'giant' type. In the present experiments platinum electrodes recorded only cells with receptive-field centre diameters of 0·5 mm (2°) or more, in agreement with the notion that electrode size, cell size and measured receptive field sizes are related. Such a relation is suggested by

the predominance of small cell bodies and small receptive-field centres in the area centralis. Micropipettes with very fine tips showed less selectivity and accordingly recorded cells with a range of centre sizes from 0·125 mm (0·5°) to 2 mm (8°). The selectivity of different electrodes is also reflected by the finding that among fifty-six ganglion cells recorded in the area centralis with platinum electrodes there were three times more 'on' centre than 'off' centre units, whereas micropipettes, in the same area, recorded ganglion cells of the two types in about equal numbers.

SUMMARY

1. Ganglion-cell discharges were recorded in the intact eye with micropipettes and platinum electrodes. Receptive fields of ganglion cells were studied in the light-adapted state mainly by measuring the relation of threshold to area of illumination.

2. For different ganglion cells receptive-field centres varied in diameter from 0·125 mm (0·5°) to 2 mm (8°). With the light stimulus restricted to the centre of receptive fields the sensitivity of ganglion cell responses was not dependent on the size of the centre region.

3. Ganglion cells with small receptive-field centres showed an increase in threshold of up to 2·5 log. units when the peripheral portion of receptive fields was included in the area of illumination. This peripheral suppression of centre responses was less pronounced for large-centre ganglion cells. Thus at whole-field illumination (diffuse light stimulation) there was a clear difference in sensitivity of ganglion-cell responses depending on the size of field centres.

4. Ganglion cells with small field centres were most often recorded in the area centralis, the region for high acuity vision; larger field centres were more common for ganglion cells recorded in the periphery of the retina.

5. Micropipettes recorded activity from ganglion cells with a wide range of field-centre sizes, whereas ganglion cells with large receptive-field centres were favoured by platinum electrodes, which presumably recorded from larger diameter cells.

This work was supported by a research grant from the National Institutes of Health, United States Public Health Service.

REFERENCES

BARLOW, H. B. (1953). Summation and inhibition in the frog's retina. *J. Physiol.* **119**, 69–88.

BARLOW, H. B., FITZHUGH, R. & KUFFLER, S. W. (1957*a*). Dark adaptation, absolute threshold and Purkinje shift in single units of the cat's retina. *J. Physiol.* **137**, 327–337.

BARLOW, H. B., FITZHUGH, R. & KUFFLER, S. W. (1957*b*). Change of organization in the receptive fields of the cat's retina during dark adaptation. *J. Physiol.* **137**, 338–354.

BROWN, K. T. & WIESEL, T. N. (1959). Intraretinal recording with micropipette electrodes in the intact cat eye. *J. Physiol.* **149**, 537–562.

FITZHUGH, R. (1957). The statistical detection of threshold signals in the retina. *J. gen. Physiol.* **40**, 925–948.

GRANIT, R. (1947). *Sensory Mechanisms of the Retina*, 412 pp. London: Oxford University Press.

GRANIT, R. (1955). *Receptors and Sensory Perception*. New Haven: Yale University Press.

GRANIT, R. & SVAETICHIN, G. (1939). Principles and technique of the electrophysiological analysis of color reception with the aid of microelectrodes. *Uppsala LäkFören. Förh.* **65**, 161–177.

GRETHER, W. F. (1941). Comparative visual acuity thresholds in terms of retinal image width. *J. comp. Psychol.* **31**, 23–33.

HARTLINE, H. K. (1938). The response of single optic nerve fibers of the vertebrate eye to illumination of the retina. *Amer. J. Physiol.* **121**, 400–415.

HUBEL, D. H. (1960). Single unit activity in lateral geniculate body and optic tract of unrestrained cats. *J. Physiol.* **150**, 91–104.

HUBEL, D. H. & WIESEL, T. N. (1959). Receptive fields of single neurones in the cat's striate cortex. *J. Physiol.* **148**, 574–591.

HUBEL, D. H. & WIESEL, T. N. (1960). Receptive fields of optic nerve fibres in the spider monkey. *J. Physiol.* (in the Press).

KUFFLER, S. W. (1953). Discharge patterns and functional organization of mammalian retina. *J. Neurophysiol.* **16**, 37–68.

KUFFLER, S. W., FITZHUGH, R. & BARLOW, H. B. (1957). Maintained activity in the cat's retina in light and darkness. *J. gen. Physiol.* **40**, 683–702.

RUSHTON, W. A. H. (1949). The structure responsible for action potential spikes in the cat's retina. *Nature, Lond.*, **164**, 743–744.

SMITH, K. U. (1936). Visual discrimination in the cat. IV. The visual acuity of the cat in relation to stimulus distance. *J. genet. Psychol.* **49**, 297–313.

TALBOT, S. A. & KUFFLER, S. W. (1952). A multibeam ophthalmoscope for the study of retinal physiology. *J. opt. Soc. Amer.* **42**, 931–936.

WIESEL, T. N. (1958). Recording inhibition and excitation in the cat's retinal ganglion cells with intracellular electrodes. *Nature, Lond.*, **183**, 264–265.

WIESEL, T. N. & BROWN, K. T. (1958). Analysis of receptive fields in the cat's retina. *Ann. N.Y. Acad. Sci.* **74**, 405–406.

PROCEEDINGS OF
THE PHYSIOLOGICAL SOCIETY

UNIVERSITY LABORATORY OF PHYSIOLOGY,
OXFORD

28 *May* 1960

Peritoneal drainage into the lymphatics of the diaphragm demonstrated *in vivo* by radiography. By GWENDA R. BARER. *Nuffield Institute for Medical Research, University of Oxford*

A transistor stimulator for class practical work. By W. J. BANNISTER and R. H. KAY. *University Laboratory of Physiology, Oxford*

In elementary electrophysiology classes it is customary to repeat pioneer experiments, using induction coils for stimulation and mechanical devices to time the shocks. Nevertheless, little may be lost in substituting a simple transistor stimulator for the induction coils and temperamental contact-breakers.

Catton, Molyneux & Schofield (1957) have exhausted the possibilities of a single-transistor circuit which may not be quite adequate for this purpose. George (1959), using twenty-two transistors, has designed a very versatile stimulator, deserving the full-scale display only found in more advanced classes and in research.

The present circuit uses nine identical inexpensive transistors in easily understood circuit configurations—the transistor analogues of (*a*) a multivibrator for p.r.f. (pulse repetition frequency) control, (*b*) a flip-flop controlling pulse duration, (*c*) another flip-flop, which can be switched into circuit to trigger (*b*) with a second delayed pulse, and (*d*) a double emitter-follower output stage based on George (1959).

The over-all performance is:

Single pulses

(1) P.r.f. (stepped control): 0·3, 1, 2, 5, 10, 20, 50, 100, 500/sec.
(2) Pulse duration (stepped control): 20, 50, 100, 200, 300, 500 μsec; 1, 2, 5, 10, 25 msec.
(3) Amplitude (stepped control): two ranges 0–4 V and 0–20 V with a good quality potentiometer spanning each range.
(4) Push button: to trigger single pulses manually when desired (or a single pair of pulses as below).

Paired pulses

(5) Delay between pulses (stepped control): 0·1, 0·2, 0·3, 0·5, 1, 2, 3, 5, 10, 20 msec.

(6) Amplitudes identical.

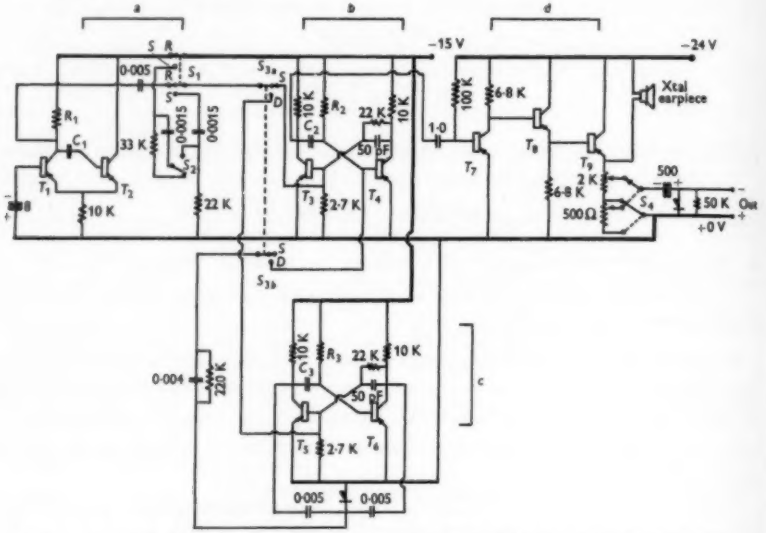


Fig. 1. T_1–T_9. Newmarket V 10/30 A: R_1C_1, p.r.f.; R_2C_2, pulse duration; R_3C_3, delay; S_1, manual-repetitive switch; S_2, push button for manual trigger; S_3, single or paired pulses; S_4, amplitude range. (R_1 = 100 K, C_1 = 0·5 μF for 0·3/sec; R_2 = 220 K, C_2 = 0·2 μF for 25 msec; R_3 = 100 K, C_3 = 0·5 μF for 20 msec.)

A small deaf-aid earpiece provides audible monitoring of the output. In paired pulse operation, the triggers are the differentiated positive-going step in the T_6 collector wave form followed by the delayed, differentiated positive step at the T_5 collector. The effective output impedance during the pulse is *ca.* 500Ω. Total battery consumption is *ca.* 4 mA at 15 V and 10 mA at 24 V. Like much teaching apparatus the stimulator is not to be despised for some research problems; its usefulness and limitations are obvious. No interlocking of switches is provided to avoid incompatibles; it is perhaps valuable for the student to realize, for example, that applying pulses at p.r.f. > 1/pulse duration, or delaying by times > 1/p.r.f. or < pulse duration are likely to confuse the experimental results. The total component cost is less than that of one induction coil and no springs or pendulums are needed.

REFERENCES

CATTON, W. T., MOLYNEUX, L. & SCHOFIELD, B. (1957). *Electron. Engng*, **29**, 496–498.

GEORGE, R. E. (1959). *Electron. Engng*, **31**, 530–533.

Recording the discharge of a single muscle-spindle afferent by means of a dekatron scaler. By R. J. HARVEY,* R. H. KAY and P. B. C. MATTHEWS. *University Laboratory of Physiology, Oxford*

In investigating the response of mammalian muscle spindles to slow extension, the discharge of a single afferent is being recorded from a dorsal-root filament and displayed on the static spot of a cathode-ray tube which is photographed on moving paper. In order to be able to measure the frequency of discharge by counting the individual impulses the paper speed has to be rather high. A slower paper speed can be used, however, by simultaneously recording on the second beam of the cathode-ray tube the output of a dekatron scaler (Ericsson, type 101A) which is triggered by the amplified action potentials and which gives a pulse for every tenth impulse. Such display both saves recording paper and reduces the labour involved in measuring the records, yet sacrifices little information when the rate of discharge of the spindle is not changing rapidly.

A thermo-electric 'cold' stimulator. By G. GORDON and R. H. KAY. *University Laboratory of Physiology, Oxford*

The thermo-electric effect is reversible—a current passed through a thermocouple produces a temperature difference between the two junctions; and if the appropriate junction is kept at constant temperature the other will cool down. Unfortunately the over-all Ri^2 joule heating overcomes the Peltier effect ($\propto i$) in the usual metallic thermocouples, at all but very small currents.

A figure of merit for the Peltier effect contains a term $\alpha^2\sigma/K$, where α is the thermoelectric power, σ the electrical conductivity and K the thermal conductivity; and recent solid-state research has developed semiconductors in which the ratio α^2 to σ/K is larger than in conventional thermocouple metals by 10 to 100 times. This outweighs a decrease in σ/K sufficiently to allow much higher currents to be passed, with greater cooling, before the over-all joule heating becomes excessive. The principal industrial application is in refrigeration (Joffe, 1957; Wright, 1959); but a small-scale physiological application would be the construction of electrically controlled 'thermodes'.

The two materials of the couple we used are *p*- and *n*-forms of a solid solution between Bi_2Te_3 and Sb_2Te_3: the alloying process reduces K with little effect on σ, and α is high. When currents of *ca.* 10 A are passed and the 'hot' face is kept at constant temperature by loading with a mass of copper, the 'cold' face falls in temperature, approximately exponentially,

* M.R.C. Scholar.

by *ca.* 10° C (90% effect in 20 sec). More important for a 'thermode', the initial rate of temperature fall is reasonably fast—*ca.* 1° C/sec mean over the first 5 sec after switching on. The final lowest temperature and the initial rate of fall increase with increasing current until the joule heating again becomes important (Fig. 1*a*); they depend also on the thermal loading of the cold junction.

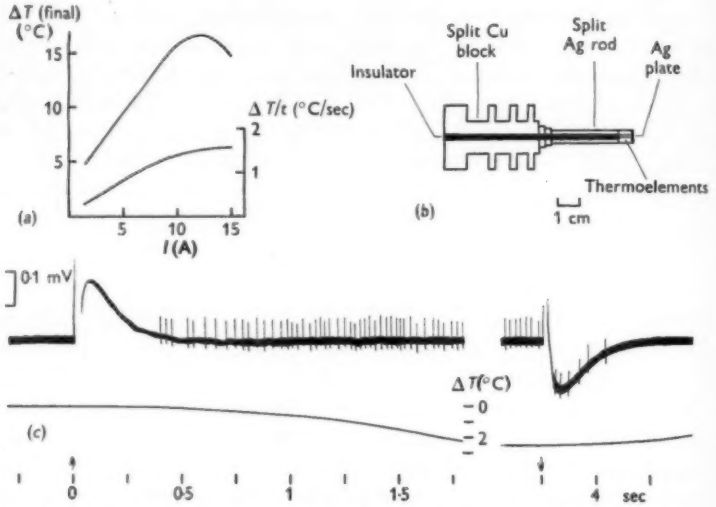


Fig. 1. (*a*) Shows changes in greatest temperature fall (upper curve, left ordinate scale) and in mean rate of temperature fall during first 5 sec (lower curve, right ordinate scale), as function of current flow. Thermode unloaded (in air). (*b*) Diagram of thermode to show dimensions. (*c*) Tracing of discharge in 'cold fibre' produced by thermo-electric cooling. Fall in temperature (ΔT), lower trace: temperature at time 0, approx. 24° C. Cat: pentobarbitone sodium anaesthesia.

The instrument to be demonstrated has proved to be simple and effective when used to stimulate the receptive areas of typical single 'cold fibres' (Hensel & Zotterman, 1951) from the cat's lingual nerve. A quite rapid response was obtained (Fig. 1*c*), the thermode being loaded by the cat's tongue.

The advantages of the thermo-electric thermode result from the greater flexibility of electrical control compared with liquid streams, giving the possibility of using several thermodes or combinations of thermal and electrical stimuli, timed with respect to one another and to an oscilloscope sweep. The device could respond to predetermined stimulus patterns. The resting temperature of the cold junction in contact with the tissue

may be held steady either above or below tissue temperature by biasing with a small constant current flowing in the appropriate direction, the larger current step to produce cooling then being superimposed. Alternation between heating and cooling stimuli depends merely on current reversal.

Disadvantages of the instrument are that it is unlikely to achieve the very rapid temperature changes possible with rapidly flowing water streams; and that by the nature of the physiological experiment the thermode lies near the recording site, where the instantaneous initiation or cessation of the large direct current flow produces an artifact at the recording electrodes (Fig. 1*c*). This artifact may sometimes interfere briefly with the dynamic phase of the recorded response; and it will probably be difficult to reduce it very greatly.

A full technical description will be published later.

We wish to thank G.E.C., Wembley, for their kindness in supplying some pieces of *p*- and *n*-type material, and particularly Dr R. Smith and Professor D. A. Wright of the research department who arranged the gift.

REFERENCES

HENSEL, H. & ZOTTERMAN, Y. (1951). *Acta physiol. scand.* **23**, 291.
JOFFE, A. F. (1957). *Semiconductor Thermoelements*. London: Infosearch.
WRIGHT, D. A. (1959). *Electron. Engng*, **31**, 659.

The effect on corneal nerve fibres of a reduction in tissue osmotic pressure. By K. C. AGARWAL and F. C. RODGER. *Institute of Ophthalmology, Aligarh University, India, and University Laboratory of Physiology, Oxford*

The non-beaded appearance of nerve fibres has only recently been confirmed by tissue culture studies.

That the beaded appearance might be an artifact was first proposed independently by Weddell & Zander (1951) and Rodger (1951*a*). In a later paper, Rodger (1951*b*) said 'there seems every reason to conclude that beading is but evidence of the response of a nerve fibre to an environmental change, physico-chemical or irritant in nature'. Using the phase-contrast microscope, we attempted to discover which of several probable factors was responsible for beading.

Fresh corneal slices were excised from rabbit eye with a Graefe knife and mounted under the phase microscope in isotonic saline at 37° C. The slice was placed epithelial side down; thus presumably the saline in which it floats is able to diffuse easily into the stromal spaces.

On irrigating such fresh specimens with isotonic saline at temperatures ranging up to 42° C, we found no evidence of our having induced beading. In a similar way fresh slices were treated with isotonic saline solutions,

the pH's of which had been altered by using phosphate buffers ranging from 4·8 to 7·2; shrinkage but no beading appeared. However, when isotonic saline was replaced by distilled water, the nerve fibres, which a moment previously had been smooth, became freely and uniformly beaded; moreover, the pointed smooth free-endings in the epithelium of the cornea became swollen until they resembled the specialized end-bulbs described by Wolff (1954). Apparently, then, nerve-fibre beading in the cornea depends upon a reduction in the osmotic pressure of the tissue fluid diffusing through the cornea and suffusing the nerves. The rotund nature of the artifact produced at the end of the terminal corneal fibres suggests that similar-looking end-organs elsewhere should be suspect, but has no bearing on other specialized endings.

After death the resultant depression of the metabolism of the nerve sheaths almost certainly results in loss of their ability to exclude sodium and chloride ions, so that after a while beading appears even where the irrigating fluid is isotonic. This can be largely prevented by irrigating with hypertonic saline.

REFERENCES

Rodger, F. C. (1951*a*). *Trans. Ophthal. Soc. U.K.* **71**, 687.
Rodger, F. C. (1951*b*). *J. Physiol.* **115**, 67*P*.
Weddell, G. & Zander, E. (1951). *J. Anat., Lond.*, **85**, 242.
Wolff, E. (1954). *Anatomy of Eye and Orbit*, 4th edition, p. 40. London: H. K. Lewis and Co.

Continuous superfusion by means of a simple roller pump.

By O. B. Saxby, S. Siddiqi and J. M. Walker. *Department of Pharmacology, University of Oxford*

Vane (1958) showed that the blood-bathed isolated organ was a convenient preparation for testing the circulating blood for active substances. The following is a description of a simple method of continuously superfusing an isolated organ with blood by means of a roller pump.

The roller pump *A* is connected to a three-way Perspex tap *B* so that blood can be taken to the superfused organ from either the carotid artery *C* or the external jugular vein *D*. A second tap *E* can be turned so that the jugular blood can flow freely from the head to the heart when it is not being used for superfusion. When the blood has passed over the isolated organ, it is collected and taken by a tube, which runs side by side with the first tube under the rollers of the pump, into a reservoir *F* which acts as an air trap. Thence it is returned to the animal through a cannula *G* in the left external jugular vein. The volume of blood outside the animal is from 5 to 6 ml. The speed at which the pump rotates can be adjusted so that blood is delivered at the desired rate.

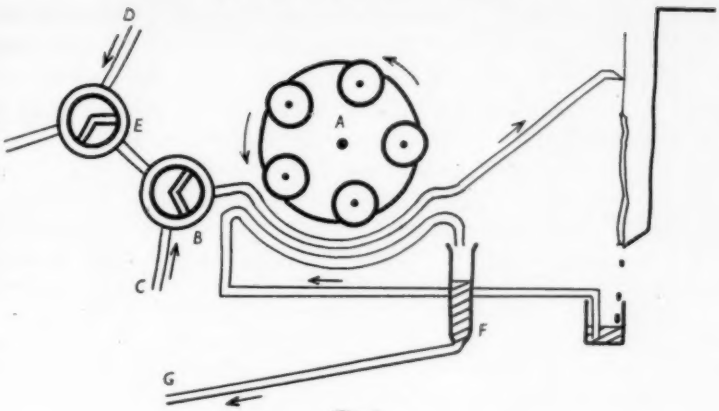


Fig. 1

The method can be applied to various experimental animals including rats. It can be used to superfuse any suitable isolated organ with blood from any convenient artery or vein.

REFERENCE

VANE, J. R. (1958). *J. Physiol.* **143**, 75–76 *P*.

A simple spirometer modification for recording volume changes electrically. By B. SMALLEY. *Department of Applied Physiology, London School of Hygiene and Tropical Medicine, W.C.*1

Inspired and expired volumes may be measured by a spirometer or, when fast transients are required, by integration of flow measured on a pneumotachograph. An electric signal has, in the past, been obtained from the spirometer by an accurate potentiometer actuated by the spindle of the spirometer wheel and also by contacts mounted peripherally on the wheel.

The principle of the device is the triggering of a photocell by the movement of the spirometer wheel. The wheel, made of Perspex, is painted an opaque black and the rim has been graduated by etching away the paint so as to produce translucent graduations at 100 ml. intervals. The light source and photocell are mounted on either side at the edge of the wheel so that light reaches the cell through the graduations. For greater convenience, provision has been made for each 500 ml. to produce an increased signal.

The use of the device is demonstrated by the measurement of non-uniformity of ventilation by a single-breath test.

The effect of removal of glucose from the medium on the electrical excitability of smooth muscle. By J. Axelsson and Edith Bülbring. *Department of Pharmacology, University of Oxford*

Feldberg & Solandt (1942) showed that removal of glucose from the medium abolishes the tone and the spontaneous contractions of isolated intestinal smooth-muscle preparations. We found recently (Axelsson, Bueding & Bülbring, 1959) that this relaxation is not due to the cessation of the spontaneous discharge of action potentials, which continues for many hours in the absence of glucose, but to the failure of the action potentials to evoke a tension response. At the same time the membrane becomes more excitable. Sometimes, but not always, the rate of spontaneous spike discharge increases. The range of frequencies at which the preparation can be driven by electrical stimulation becomes wider and increases particularly towards the higher frequencies.

When the normal glucose concentration is restored, spontaneous activity slows and may stop. The range of frequencies at which the preparation can be driven becomes narrower and declines towards the lower frequencies.

These excitability changes in the absence and presence of glucose will be demonstrated on the isolated taenia coli preparation of the guinea-pig.

REFERENCES

Axelsson, J., Bueding, E. & Bülbring, E. (1959). *J. Physiol.* **148**, 62–63*P*.
Feldberg, W. & Solandt, O. M. (1942). *J. Physiol.* **107**, 137–171.

The primary and secondary endings of the mammalian muscle spindle. By D. Barker and M. C. Ip. *Department of Zoology, University of Hong Kong*

The presence of two kinds of sensory endings in the equatorial region of the mammalian spindle was established by Ruffini (1898) who proposed for them the terms 'primary' and 'secondary' together with the descriptive equivalents 'annulo-spiral' and 'flower-spray'. However, the 'flower-spray' secondary in the cat is less common than the secondary which consists chiefly of rings and spirals. In a sample of thirty-two intermediate, twenty-five complex, and four tandem spindles from rectus femoris there were ninety-eight secondary endings; 64 % consisted chiefly of rings and spirals (*S* type) and 36 % chiefly of sprays (*s* type). This finding, together with variation in regularity of the primary ending, suggests that the form of the terminal ramifications of both endings is related to the morphology of the muscle fibre in the region of termination. The extremes of regularity and irregularity are displayed on the one hand by the rings and spirals of the primary around nuclear bags, and on the other by the sprays of

the secondary on polar-form muscle fibres. While the diameter of the nerve fibre supplying the primary is larger than that of the secondary, and the width of its terminal ramifications greater, there is little difference in form between a primary and a secondary ending, or between portions of such endings, when they are associated with myotubes; in both cases the configuration is 'annulo-spiral'. The nature of the intrafusal bundle is such that nuclear bags, myotubes, and polar-form fibres may be present in the equatorial region in any combination at almost any level. Hence

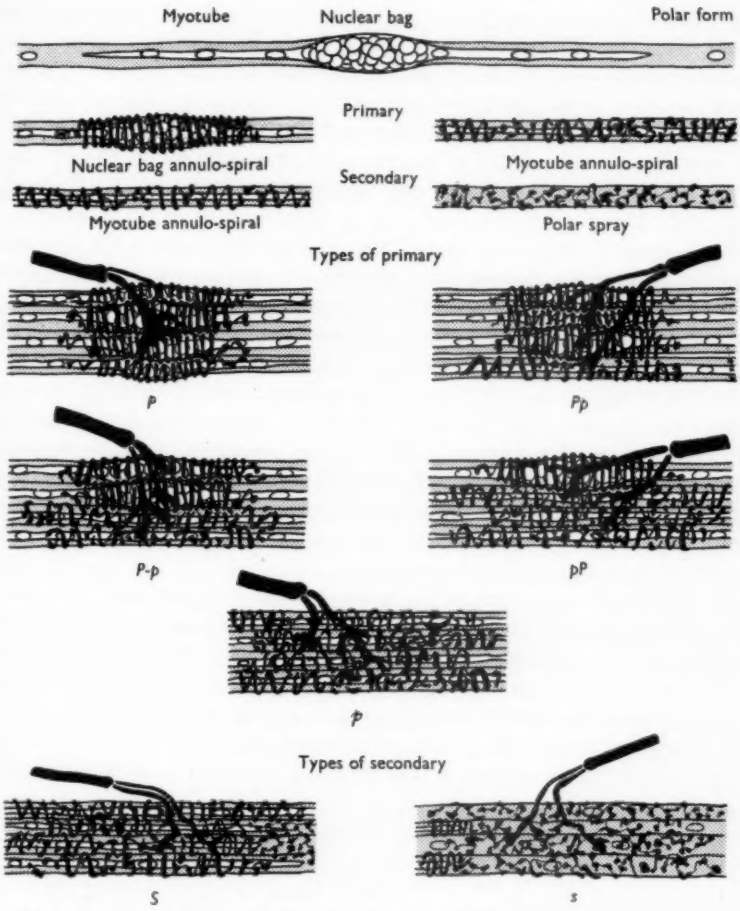


Fig. 1. Variation in form of mammalian primary and secondary endings. Cross-striations of muscle fibres omitted; symbols as in text.

various combinations of regularity and irregularity are presented by the two endings where their ramifications come into relation with the individual members of the bundle (Fig. 1). The matter is further complicated by the fact that though the area of the secondary ending usually lies adjacent to that of the primary, it may extend over certain members of the bundle so as to lie partly alongside it.

The regularity of the primary ending depends on the ratio of nuclear bags to myotubes in the region of termination. Since the intrafusal bundle here is usually a mixture of each, and is seldom composed exclusively of one or the other, it is possible to distinguish between five types of primary: wholly regular (*P*); wholly irregular (*p*); predominantly regular (*Pp*); predominantly irregular (*pP*); about half and half (*P-p*). The predominantly regular type is the most common; in a sample of 155 cat spindles comprising 166 capsular regions (seven spindles from flexor digiti longus, the rest from rectus femoris; fourteen tandem spindles in the sample), 50·5 % of the primaries were of the *Pp* type; 17 % *pP*; 15·7 % *P*; 10·8 % *P-p*; and 6 % *p*.

The greater frequency of the *S* type of secondary reflects the fact that the ramifications are more often in association with myotubes (as rings and spirals) than polar-form muscle fibres (as sprays). It is possible that in his anxiety to establish the existence of the secondary ending, then in dispute, Ruffini exaggerated its 'flower-spray' features. In his classic paper (1898) it is stylized wholly in this form in the schematic Fig. 1, and Fig. 4 shows a drawing of typical polar sprays. A survey of spindle afferent endings as illustrated in the literature for various mammals, and as available to us in opossum, bat, rabbit, rat, cat, and monkey preparations, indicates that while there are minor variations in form between species, the major factor determining rings, spirals, and sprays is the morphology of the muscle fibre in the region of termination.

We suggest that the terms 'annulo-spiral' and 'flower-spray' be abandoned as equivalents for the primary and secondary endings of a spindle. To refer to the primary as a 'nuclear bag ending' and the secondary as a 'myotube ending', as suggested by Granit (1955), is also inappropriate. 'Primary' and 'secondary' are adequate terms in every respect since they accurately represent the histological, embryological, and physiological characteristics of the endings and nerve fibres (Groups I a and II) concerned.

This investigation was supported by a research grant (B-1806) from the National Institute of Neurological Diseases and Blindness, U.S. Public Health Service.

REFERENCES

GRANIT, R. (1955). *Receptors and Sensory Perception*. New Haven: Yale University Press.
RUFFINI, A. (1898). *J. Physiol.* **23**, 190–208.

The estimation of sulphobromophthalein (BSP) in turbid and grossly haemolysed plasma and in liver. By W. H. H. ANDREWS and MARGARET TRENT. *Department of Pharmacology, St Mary's Hospital Medical School, London, W. 2*

The estimation of BSP in moderately cloudy or haemolysed plasma can be carried out by measuring absorption at two wave-lengths (Gaebler, 1945) but this procedure fails when the haemolysis or cloudiness is gross. The acetone extraction method of Brauer, Pessotti & Krebs (1954) is not accurate unless the evaporation of acetone is carefully controlled, and the development of colour is poor, presumably owing to incomplete ionization of BSP in acetone. The following method, though somewhat tedious, has been found to be accurate to within 8 % and is usually accurate to within 5 % even in the presence of large amounts of haemoglobin.

Plasma, 0·5 ml. mixed with 0·5 ml. distilled water, is added slowly, with shaking, to 2 ml. acetone in a centrifuge tube. In preparation of standards the plasma is diluted with BSP solution. The mixture is then centrifuged for a constant rate for a constant time (1700 rev/min for 15 min) to keep evaporation fairly constant in all tubes.

Two ml. of the supernatant fluid is pipetted into a 10 ml. test-tube, graduated at 4 ml., and the acetone is driven off by heating at 70° C for 15 min, preferably in a partial vacuum with an air leak. After the fluid is cool, 0·5 ml. 'Liquid Lux' is added together with distilled water to 4 ml. Lastly 1 drop of aqueous sodium hydroxide, approximately 30 %, is added. The solution becomes turbid but clears on standing for about an hour. The BSP is estimated by comparison with standards at 580 mμ.

For estimation of BSP in liver, 0·5 g tissue and 0·5 ml. distilled water are ground together with sand and then washed into a graduated centrifuge tube with four successive washings each of about 1·5 ml. of an acetone–water mixture (3 vol. acetone to 1 vol. water). The contents of the tube are made up to 10 ml. by addition of the mixture and centrifuged at 1700 rev/min for 15 min. Four ml. of the supernatant fluid is placed in a 10 ml. tube, graduated at 4 ml., and evaporated at 70° C as above. The rest of the procedure is as with plasma.

Recovery of BSP added to homogenized liver is 80–90 %, or even greater, in the range of concentrations 1–16 mg BSP/100 g liver tissue. Recovery of BSP from liver tissue in the only other published method which the authors have been able to find (Richards, Tyndall & Young, 1959) appears to be very much less than 70 %. Blank readings at 580 mμ obtained when rat liver was extracted without added dye were equivalent to 0·25 mg BSP/100 g liver tissue or less.

'Liquid Lux' is a commercial product, manufactured by Lever Bros. Ltd., and is a mixture of various detergents, the composition not being publicly disclosed.

We are grateful to Dr T. G. Richards for assessing the accuracy of the method when applied to plasma.

REFERENCES

BRAUER, R. W., PESSOTTI, R. L. & KREBS, H. A. (1955). *J. clin. Invest.* **34**, 35–43.
GAEBLER, O. H. (1945). *Amer. J. clin. Path.* **15**, 452–455.
RICHARDS, T. G., TYNDALL, V. R. & YOUNG, A. (1959). *Clin. Sci.* **18**, 499–511.

Pharmacologically active substances in a caecal sac of the male Garden Tiger moth. By E. H. LEACH, J. A. PARSONS and W. D. M. PATON. *Department of Pharmacology, University of Oxford*

A simple method for restraining rabbits during pyrogen assays. By A. J. OUGH. *M.R.C. Group for Research on Body Temperature Regulation, Department of the Regius Professor of Medicine, The Radcliffe Infirmary, Oxford*

An instrument for charting the brain surface. By C. G. PHILLIPS and E. H. J. SCHUSTER. *University Laboratory of Physiology, Oxford*

Aid to alignment of catheter for insertion into abdominal vessels. By PEGGY J. COSGROVE, R. V. COXON and ANN SPENCER. *University Laboratory of Physiology, Oxford*

Sites of cholinesterase activity in cultured chick-embryo skeletal muscle cells. By W. K. ENGEL. *The National Institutes of Health, Bethesda, Maryland, U.S.A.*

Responses of single cells in the caudal part of the spinal nucleus of the trigeminal nerve of the cat. By G. GORDON, S. LANDGREN and W. A. SEED.* *University Laboratory of Physiology, Oxford*

The spinal nucleus of the trigeminal nerve extends from the rostral medulla to approximately the second cervical segment, and may be regarded as containing the trigeminal equivalent of the cells of the 'spinothalamic' system. Medullary section of the afferent fibres to this nucleus in man causes facial analgesia with impairment of thermal sense but

* M.R.C. Scholar.

preservation of tactile sense (Sjöqvist, 1938). In the present investigation, limited to the caudal part of the nucleus, responses of single cells were recorded with KCl-filled micropipettes, and mechanical or thermal stimuli were applied to the ipsilateral facial area. The cats were anaesthetized with pentobarbitone sodium.

The majority of cells responded at low threshold to mechanical stimuli applied to hairs, skin, or mucous membrane, without significant thermal sensitivity. The responses were of a rapidly-adapting character, and receptive areas were well defined. Many such cells were fired antidromically by stimulating the contralateral medial lemniscus region. Electrical stimuli to the receptive area caused responses at 1·8–4·0 msec latency. There was a well-marked and orderly topographical organization of these cells which agrees with anatomical evidence—mandibular division lying medial, ophthalmic division lateral, and maxillary division in between. Receptive areas varied for different cells, the largest being some 25 cm^2 and the smallest confined to a group of several short hairs or to a single maxillary whisker. On a number of occasions responses to mechanical stimulation of a whisker were inhibited by mechanical conditioning stimuli applied to other whiskers or nearby hairs.

A second group of deep-lying cells had functional characteristics distinguishing them from the first group. Their receptive areas were large, surpassing the boundaries between the peripheral divisions of the trigeminal nerve and occupying much of the face. They responded to mechanical pressure with sustained discharge, threshold normally being lowest in the centre of the receptive field where light pressure was usually effective. In the more peripheral parts of their receptive fields firm pressure with a glass rod, light pinches, or pinpricks were effective stimuli for average cells; and where applied steady pressure could be satisfactorily measured (forehead, pinna, or tongue), thresholds were of the order of 10^6 dynes/cm^2. The discharge usually outlasted the stimulus by a few seconds, with gradual reversion to a base line of 'spontaneous' activity. These cells also responded to large thermal stimuli (over 54° C or below 10° C). Minimal latencies of response to cutaneous electrical stimuli lay between 2 and 15 msec. Only one of the twenty cells examined was fired antidromically from the lemniscus. Their responses to skin stimulation were unaffected when neuromuscular block was induced by giving 8 mg gallamine triethiodide, anaesthesia being maintained at a steady level.

No cells were encountered which showed any appreciable response to thermal skin stimuli within the range 10–45° C.

REFERENCE

SJÖQVIST, O. (1938). *Acta psychiat., Kbh.*, Suppl. **17**, 1.

Bronchial circulation and pulmonary vascular resistance (PVR) in isolated perfused lung lobes. By I. DE BURGH DALY and B. A. WAALER. *University Laboratory of Physiology, Oxford*

There is little information of the progressive changes in PVR which occur in isolated lungs during perfusion periods of several hours. In order to follow these changes perfusions were carried out in isolated lung lobe preparations of the dog. The perfusate was autologous blood and the method for perfusion a modification of that previously described by Daly (1956). Either constant volume inflow or constant pressure inflow perfusion was used. The tidal air was measured. For the calculation of the PVR for both lungs the following formula was used

$$\text{PVR} = \frac{\text{Pulmonary arterial pressure (mm Hg)} - \text{Left atrial pressure (mm Hg)}}{\text{Left atrial flow (l./min)} \times x/\text{m}^2},$$

where x equals the ratio of total lung weight to the weight of the perfused lobes, as given by Rahn & Ross (1957). When this formula is used, the PVR for normal resting dogs ranges from 1·7 to 7·5.

In one group of seven experiments, in which the pulmonary circulation alone was perfused, the PVR values dropped markedly during the first 2 hr of perfusion to a mean of 4·9 and remained stable at this level for periods up to 7 hr of perfusion. In another group of seven experiments, where in addition the bronchial circulation was perfused, markedly higher PVR values developed. The mean value dropped to 6·7 after 1½ hr of perfusion and thereafter showed a slow but steady increase. The bronchial circulation perfusion did not influence the long-term progressive changes in tidal air.

Some handling of the lung lobes is inevitable during the preparation for a perfusion experiment, even if great care is taken to avoid it. It was suspected that handling might influence the PVR on subsequent perfusion. In order to test this, gentle manipulation or stroking of the lobes before starting perfusion was carried out in a separate group of experiments. Such handling did not affect the PVR when subsequently the pulmonary circulation alone was perfused, but caused a severe rise in PVR when in addition the bronchial circulation was perfused.

REFERENCES

DALY, I. DE B. (1956). *J. Physiol.* **132**, 42–43.
RAHN, H. & ROSS, B. B. (1957). *J. appl. Physiol.* **10**, 154–157.

Glycolysis and potassium transport in kidney slices from new-born animals. By R. WHITTAM. *Medical Research Council Cell Metabolism Research Unit, Department of Biochemistry, University of Oxford*

Slices of the cortex cut from the kidneys of adult rabbits, rats and guinea-pigs gain sodium and lose potassium during anaerobic incubation, because they depend on respiration for the maintenance of concentration gradients of these ions between intra- and extracellular fluids (Mudge, 1951; Aebi, 1953; Whittam & Davies, 1953). Adult animals, and also new-born guinea-pigs, do not tolerate anoxia for as long as new-born rabbits and rats (Fazekas, Alexander & Himwich, 1941), suggesting that the latter might be able to prevent leakage of potassium from the tissues into the circulation, which appears to be of some importance to survival (Dawes, Mott & Shelley, 1959). This possibility has been tested by measuring the potassium concentration in kidney cortex slices from new-born and adult rabbits, rats and guinea-pigs after incubation under various conditions. The rates of glycolysis were also measured as indications of the supply of metabolic energy.

The potassium concentration in kidney cortex slices from new-born rabbits and rats was about 85 m-mole/kg wet wt. after aerobic incubation for 1 hr at 37° C in a saline medium containing 168 mM-Na, 5 mM-K and 10 mM-glucose. After either aerobic incubation with 1 mM-KCN, which inhibited respiration, or after anaerobic incubation, the concentration was 25% lower, viz. 65 m-mole/kg wet wt. When 1 mM-iodoacetate was added to inhibit glycolysis, the potassium concentration after anaerobic incubation was reduced further to about 30 m-mole/kg wet wt. Abolition of respiration by either cyanide or anoxia therefore caused a smaller leakage of tissue potassium (20 m-mole/kg wet wt.) than inhibition of both respiration and glycolysis (55 m-mole/kg wet wt.). In contrast, kidney slices from new-born guinea-pigs contained the same amount of potassium (about 20 m-mole/kg wet wt.) after anaerobic incubation, in both the presence and absence of iodoacetate, and resembled the tissue from adult guinea-pigs in requiring O_2. All the leakages of potassium were approximately balanced by gains of sodium.

The rates of liberation of CO_2 from a bicarbonate saline medium (Q_{CO_2} = μl./mg dry wt. × hr) were 22 and 16 anaerobically for rabbit and rat tissue, respectively, and 3·5 aerobically. The rates for new-born guinea-pig tissue were 5 anaerobically and less than 1 aerobically.

The results show that kidney cortex slices from new-born rabbits and rats actively maintained high potassium concentrations by energy derived from glycolysis when respiration was abolished either by cyanide or anoxia.

In contrast, the same tissue from new-born guinea-pigs depended almost wholly on respiration. These relationships are compatible with the high rate of anaerobic glycolysis in the tissue from young rabbits and rats and with the resistance of these animals to anoxia.

REFERENCES

AEBI, H. H. (1953). *Helv. physiol. acta*, **11**, 96.
DAWES, G. S., MOTT, J. C. & SHELLEY, H. J. (1959). *J. Physiol.* **146**, 516.
FAZEKAS, J. F., ALEXANDER, F. A. D. & HIMWICH, H. E. (1941). *Amer. J. Physiol.* **134**, 281.
MUDGE, G. H. (1951). *Amer. J. Physiol.* **165**, 113.
WHITTAM, R. & DAVIES, R. E. (1953). *Biochem. J.* **55**, 880.

Prolongation of survival in the anoxic foetal lamb. By G. S. DAWES, JOAN C. MOTT and ANNE STAFFORD. *The Nuffield Institute for Medical Research, University of Oxford*

Previous experiments on foetal anoxia have suggested that the glycogen stores available and the fall of blood and tissue pH might be limiting factors to the continued production of energy by anaerobic glycolysis, and hence to survival. Therefore experiments were undertaken on lambs of 74–91 days gestation age, delivered by Caesarean section under chloralose anaesthesia. Asphyxia was produced by tying the umbilical cord. Infusion of glucose alone did not increase survival as judged by the blood pressure and heart rate, nor did infusion of sodium carbonate alone, in a dose which prevented any considerable fall of arterial blood pH. However, infusion of glucose, together with sodium carbonate, increased the time taken for the blood pressure to fall, from the initial level of 30–40 mm Hg down to 10 mm Hg, from a mean of 48 min to more than 80 min. Lactate production was also increased. Tris-hydroxymethylaminomethane or triethanolamine were substituted for sodium carbonate with equal effect.

Mature foetal lambs of 140–145 days gestation age were delivered by Caesarean section under local anaesthesia, a saline-filled rubber bag was placed over the head and the umbilical cord was tied. After 10–12 min asphyxia the lambs were resuscitated by artificial ventilation through an endotracheal catheter. In five sets of twins, one of each pair was untreated and the other was given an intravenous infusion of a solution containing 15% glucose and 15% sodium carbonate (0·14–0·18 ml./kg.min). At the end of 10 min asphyxia the mean rise in blood lactate was 74% greater in treated lambs. The blood pressures and heart rates of the treated lambs were all greater than those of their untreated twins. The duration of gasping, after the onset of asphyxia, was greater in the treated lambs by a mean of 27%. The recovery of the treated lambs after asphyxia was

also more rapid. All the lambs exhibited respiratory distress for a considerable period after resuscitation. There is as yet no evidence that this form of treatment will prevent permanent cerebral damage from anoxia at birth, but so far as measurements on the breathing and circulation are concerned there was some improvement.

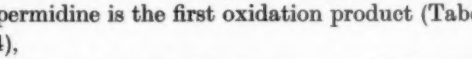
Release of noradrenaline from artery walls as a cause of gangrene. By J. H. Burn and D. McDougal. *University Laboratory of Physiology, Oxford*

Primary and secondary amines as substrates of amine oxidases. By H. Blaschko. *Department of Pharmacology, University of Oxford*

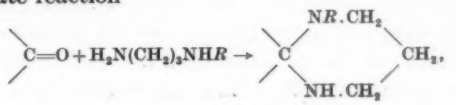
The amine oxidases of non-ruminant plasma differ from those of ruminants in that they are without significant activity on spermine. Although the substrate specificity of all the plasma oxidases is in some ways similar to that of the intracellular 'monoamine' oxidase, they resemble histaminase in being inhibited by carbonyl reagents. It is assumed that a carbonyl group in the active centre of the plasma oxidases reacts with the amino group of the substrate as shown

It might therefore be expected that these enzymes will oxidize primary amines but not secondary amines. This is in fact so; it has now been found that the plasma enzymes, ruminant and non-ruminant, readily oxidize benzylamine, β-phenylethylamine and γ-phenylpropylamine, but not the corresponding *N*-methyl derivatives. This differentiates the plasma enzymes sharply from the intracellular amine oxidase; the latter acts on substances like adrenaline or metanephrine as readily as on noradrenaline or normetanephrine. On the other hand, histaminase does not act on secondary amines (Goryachenkova, 1956; Kapeller-Adler & Iggo, 1957).

It is known that in the enzymic oxidation of spermine by ox plasma enzyme spermidine is the first oxidation product (Tabor, Tabor & Rosenthal, 1954),



This reaction involves an attack at a secondary amino group. It is suggested that spermine oxidase is able to form a ring compound in the enzyme-substrate reaction



b

and that it is the ability to form such a compound that distinguishes spermine oxidase from similar oxidases without action on spermine.

I am grateful to the Agricultural Research Council for financial help.

REFERENCES

GORYACHENKOVA, E. V. (1956). *Biochemistry, Leningr.* **21**, 322.
KAPELLER-ADLER, R. & IGGO, B. (1957). *Biochim. biophys. acta*, **25**, 394.
TABOR, C. W., TABOR, H. & ROSENTHAL, S. M. (1954). *J. biol. Chem.* **208**, 645.

Effect of pressure on heart and perfusion rates of the Langendorff preparation. By J. A. BARCLAY, E. J. HAMLEY and HELGA HOUGHTON. *Physiology Department, Birmingham University*

In rat hearts, isolated and perfused as Langendorff preparations on oxygenated Ringer–Locke solution (plus 0·2% glucose; temp. 30–35° C), heart and perfusion rates vary with perfusion pressure. Pressures below 60 cm did not always maintain normal beat and rate. At 15 cm 25%

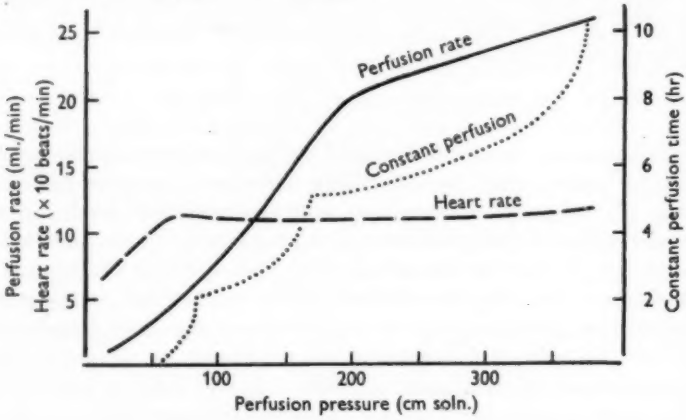


Fig. 1

of preparations failed to beat and most others developed abnormal conduction patterns within 2 hr. Above 60 cm heart rates varied little from one preparation to another; mechanical activity appeared normal and the electrogram was unchanged from what can be found in the open chest of the rat. Between 60 and 200 cm perfusion rates increased directly with perfusion pressure, but heart rate showed no similar increase and was little changed at pressures above 100 cm. At all pressures the perfusion rate had decreased to about half of original rate (taken after 5 min perfusion) in 2 hr, and tended to follow this rate of decrease as perfusion con-

tinued. Heart rate also fell but was more variable, the decrease in 2 hr being only 30% in most cases and 75% in others. Initial pressure and temperature of perfusate affected the preparation markedly. Above 30° C good cardiac activity was difficult to maintain at pressures below 60 cm; below 30° C the resulting slower beat could usually be maintained at 30 cm. Deterioration at all pressures was seldom evident at once; many preparations showed little change within the first hour but thereafter steadily decreased. Deterioration could be transiently reversed by increasing perfusion pressure, but subsequent deterioration could be corrected by further small increases in pressure. Too great an increase produced boosts to above earlier rates. By this method it was possible to maintain heart and perfusion rates over long periods of time. The dotted locus in the figure illustrates a typical experiment.

The latissimus dorsi muscles of the chick. By B. L. GINSBORG* and B. MACKAY. *Pharmacology and Anatomy Departments, University of Edinburgh*

Records of spontaneous electrical activity at the neuromuscular junction in skeletal muscles of the chick suggest that two types of muscle fibre occur, one focally innervated and the other with many motor end-plates (Ginsborg, 1959). Both types of fibre have now been demonstrated histologically. In the anterior latissimus dorsi only fibres with multiple end-plates, and in the posterior latissimus dorsi only focally innervated fibres, have been observed. It has also been found that in general it is only the anterior latissimus dorsi which responds to the application of 'depolarizing agents' with a sustained contracture (Fig. 1). In various other species of bird, the anterior and posterior latissimi dorsi have been found by Krüger (1950) to be composed entirely of 'Felderstruktur' and 'Fibrillenstruktur' fibres, respectively; the present observations are therefore in accordance with the suggestion (for which we are indebted to Mr A. F. Huxley) that the fibres with many end-plates in the bird might be identified with its 'Felderstruktur' fibres. The observations also accord with the idea (Krüger, 1950) that it is these fibres alone which are responsible for the characteristic contractures of avian muscle.

Recordings with external electrodes from the anterior latissimus dorsi confirm the finding (Ginsborg, 1959) that fibres with multiple end-plates respond to adequate stimulation with propagated action potentials. The small variation in latency of the response at different points along the

* Member of External Scientific Staff of the Medical Research Council.

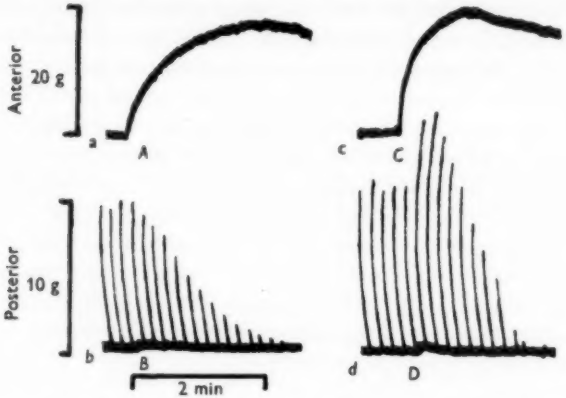


Fig. 1. Records of isometric tension (RCA 5734) from an anterior (*a*, *c*) and a posterior (*b*, *d*) latissimus dorsi muscle of a 22-day-old-chick. 37° C. Muscles in Krebs–Henseleit solution. At *A* and *B* decamethonium iodide added to give final concentrations of $1{\cdot}7 \times 10^{-6}$ M (*A*) and $3{\cdot}4 \times 10^{-5}$ M (*B*). At *C* and *D* bathing solution changed to Krebs–Henseleit with 100 mM NaCl replaced by 100 mM-KCl. In *b* and *d*, posterior latissimus dorsi nerve stimulated supramaximally at 6 min.

muscle suggests that on maximal nerve stimulation action potentials may be initiated at many, if not all, of the neuro-muscular junctions along the fibres.

REFERENCES

GINSBORG, B. L. (1959). *J. Physiol.* **148**, 50–51*P*.
KRÜGER, P. (1950). *Zool. Anz.* Erg.-Bd., **145**, 445–460.

A study of skin absorption effects of tri-ortho-cresyl phosphate (TOCP) in hens. By P. GLEES and W. G. WHITE. *University Laboratory of Physiology, Oxford*

The neurotoxic effects of TOCP ingestion are well known both in human (Smith & Spalding, 1959) and animal experiments (Barnes & Denz, 1953; Cavanagh, 1954; Glees, 1957; Silver, 1959) but far less is known of the effects of the absorption of the substance through the skin. That this can occur has been shown by applying radioactive TOCP compounds to human skin (Hodge & Sterner, 1943), and as small quantities of TOCP, or similar plasticizers, are contained in certain plastics there is a potential risk of absorption in industry.

We studied the minimal dosage of TOCP which causes either clinical or neuropathological alterations in fully grown chickens between 3 and 5 kg in weight. The substance was painted on the comb, which absorbs

it well (0·2 c.c. of pure TOCP being applied at a time). The hens were subsequently perfused with 10% formol saline to avoid the staining artifacts common in Marchi preparations of unperfused material.

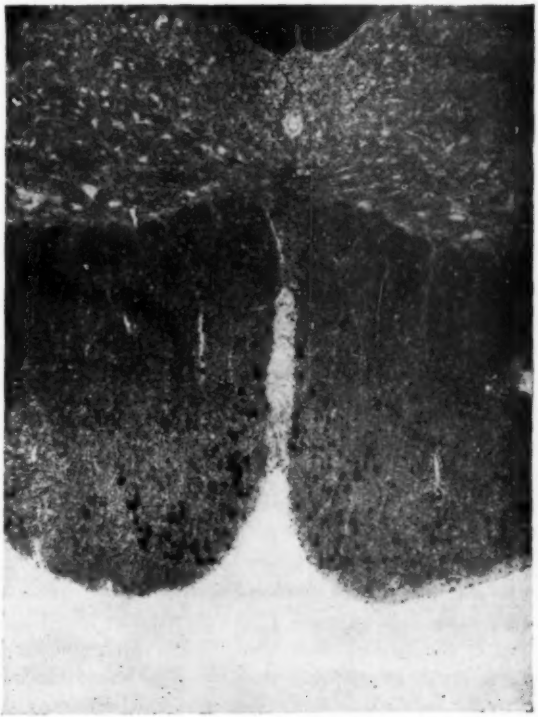


Fig. 1. Transverse section through the lumbar cord of a hen showing the Marchi positive fibres of the tecto-spinal tract. 0·05 c.c. of TOCP per 1 kg bodyweight.

We found that 0·1 c.c. of TOCP to 1 kg body weight caused, after an interval of 8–25 days, a weakness of the extensors of the legs and general symptoms of intoxication such as loss of appetite and listlessness. Half this dose caused only neuropathological changes, such as selective myelin degeneration in the long descending and ascending tracts, and the animal's behaviour and motor function gave no indication of any damage to the central nervous system, at any rate during the period we allowed for observation. Doses below 0·05 c.c. produced neither clinical nor neuropathological symptoms or poisoning, but because of the slow excretion of TOCP it is not impossible that continual small doses would in time have

the same effects as larger single doses of 0·05 c.c. Such cumulative action could result in degenerative changes in the descending and ascending tracts, ultimately presenting an obvious clinical picture. Herein, of course, lies the danger, particularly in industry, and it is a question on which further work is in progress.

REFERENCES

BARNES, J. M. & DENZ, F. A. (1953). *J. Path. Bact.* **65**, 597.
CAVANAGH, J. B. (1954). *J. Neurol.* **17**, 163–172.
GLEES, P. (1957). *Morphologie und Physiologie des Nervensystems.* Stuttgart: Thieme.
HODGE, H. C. & STERNER, J. H. (1943). *J. Pharmacol.* **79**, 225–234.
SMITH, H. V. & SPALDING, J. M. K. (1959). *Lancet*, **277**, 1019–1021.
SILVER, A. (1959). *J. Physiol.* **148**, 66*P*.

The competitive effect of probenecid on bromsulphalein uptake in rat and dog liver slices. By G. BARBER-RILEY. *Physiological Laboratory, University of Liverpool*

Investigation of the excretory role of the liver has been carried out with exogenous substances which are believed to show a part at least of the normal excretory mechanism. The most successful and generally used of these foreign substances is the dye bromsulphalein (BSP) which is almost entirely excreted in the bile.

Several substances have been shown to reduce BSP excretion, presumably by competing for the same mechanism. These substances include probenecid, but while this reduces BSP excretion in the dog, excretion of BSP in the rat is unaffected (Andrews, 1958). This species difference in the action of similar doses of probenecid on BSP excretion was confirmed in this laboratory.

BSP uptake by liver cells in dog and rat was studied by '*in vitro*' experiments with fresh liver slices incubated under controlled conditions in a standard BSP medium. In this manner the mechanism of dye uptake was artificially separated from excretion, and the probenecid effect on the uptake mechanism alone investigated.

Preliminary experiments were carried out to obtain the normal range of BSP uptake to be expected in liver slices from any individual rat. The variation in slice BSP uptake in different rats was found to be large, but the variation found in slices from the same animal was much smaller (S.D. $\pm$ 3·9 %).

Liver slices were then incubated in the standard BSP medium with added probenecid and the dye uptake measured. This figure was compared with the control BSP uptake obtained by incubating slices from the same

rat under identical conditions but without probenecid. The effect of the probenecid was to increase BSP uptake by 0·5%, a figure showing no significant difference. The same method with dog liver slices, however, showed a reduction in BSP uptake of 14·5%. Thus BSP uptake of the rat liver is, unlike the dog, not significantly affected by probenecid in the conditions of the experiment.

The above experiments were repeated using biligrafin as a competitor in place of probenecid. BSP uptake in the rat liver slices was reduced by 24% and in the dog liver slices by 27%. The competitor effect of biligrafin in these experiments is therefore substantially the same in both animals.

It therefore appears that the BSP uptake of rat liver slices is susceptible to a competitor action in the same manner as dog liver slices, but that for some reason this is not the case with probenecid. The reason for this particular species difference is uncertain at present, but the possibilities appear to include:

(1) That in the rat liver effective concentrations of probenecid do not enter the hepatic cell.

(2) That probenecid enters the cell freely in the rat liver, but is either not protein bound or occupies sites qualitatively different from BSP.

REFERENCE

ANDREWS, W. H. H. (1958). *Liver Function*, p. 128 (ed. Ralph W. Brauer). Washington, D.C.: American Institute of Biological Sciences.

The diameter and distribution of the nuclear bag and nuclear chain muscle fibres in the muscle spindles of the cat. By I. A. BOYD. *Institute of Physiology, University of Glasgow*

Normal, de-afferented and de-efferented muscles from the cat hind limb were serially sectioned transversely. The intrafusal muscle fibres were traced in seventy-eight spindles. All contained two distinct types of muscle fibre: (*a*) with numerous nuclei tightly packed together (nuclear-bag fibres), and (*b*) with a single row of nuclei down the centre (nuclear-chain fibres), in the equatorial region (cf. Cooper & Daniel, 1956). With Masson staining, the nuclear-bag fibres are uniformly dark, and the chain fibres are granular, in appearance. The chain fibres have fewer sarcostyles per unit area.

In the leg muscles the nuclear-chain fibres are much smaller in length and diameter, measured near the end of the lymph space, than the bag fibres. Two bag fibres and four or five chain fibres occur most frequently. In the small muscles of the foot the two types of fibre are sometimes about the same length and diameter, but when traced through the spindle equator, one type has nuclear bags, and the others become very small with

a row of nuclei. Spindles with up to four bag fibres and nine chain fibres have been seen.

Division of intrafusal fibres does occur (Barker, 1959), but is very rare in nuclear-bag fibres. Division of chain fibres is more common, but usually confined to one or two fibres. In some spindles all the muscle fibres are discrete. Spindles may have overlapping ends, but true tandem spindles with continuous intrafusal fibres are rare.

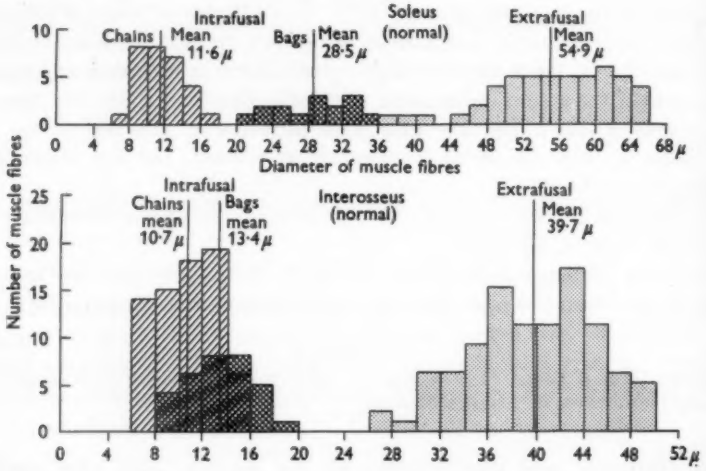


Fig. 1. Fibre-size histograms for intrafusal muscle fibres in eight spindles from soleus, and fourteen spindles from interosseus, muscles of the cat. A random sample of extrafusal fibres is included in each case. The nuclear-bag and nuclear-chain groups are distinct in soleus, and overlap in interosseus. Diameter measured in transverse sections near one end of the lymph space in Bouin-fixed, relaxed, muscle.

After ventral root section both types of intrafusal fibre show a reduction in diameter proportionately less than do the extrafusal fibres. After dorsal root section the nuclei of the equatorial region disappear in several months, the chain fibres losing their nuclei first.

I am indebted to the Rankin Fund of the University of Glasgow for defraying part of the cost of materials used in this research.

REFERENCES

Barker, D. (1959). *J. Physiol.* **149**, 7*P*.
Cooper, Sybil & Daniel, P. M. (1956). *J. Physiol.* **133**, 1*P*.

Calcium ions and the resting potential of frog sartorius fibres in 0·7% NaCl solution. By M. H. Draper, H. Friebel and K. Karzel. *Department of Physiology, University of Edinburgh, and Department of Pharmacology, University of Bonn*

Experiments on frog sartorius muscles have shown that in potassium-deficient Ringer's solution the resting potential can remain elevated for some hours (Bülbring, Holman & Lüllmann, 1956; Woodbury, 1959). We have studied the dependence of the resting potential on the external calcium-ion concentration in muscles placed in 0·7% NaCl solution (120 mM). Resting-potential determinations were carried out with micro-electrodes on pairs of frog's sartorii as described by Draper, Friebel & Karzel (1959). One muscle was kept in Ringer's solution throughout the 6 hr of the experiment and its mean resting potential was sampled hourly as a control, and the other was placed in 0·7% NaCl solution after its initial resting potential in Ringer's solution had been determined by sampling twenty fibres. Temperature was maintained at 14° C.

When frog muscle fibres were placed in 0·7% NaCl solution the mean resting potential rose from 88 to 97 mV and then after about 15 min slowly declined reaching 55 mV in 2 hr. During this time in NaCl the well-known 'salt twitches' occurred. Replacing the muscle in Ringer's solution resulted in recovery of the resting potential to 90% of its initial value in 2 hr (Draper *et al.* 1959).

The addition of Ca^{2+} increased the degree and duration of hyperpolarization. Thus the presence of 0·01 mM-Ca^{2+} resulted in a rise of the resting potential to a maximum of 102 mV (S.E. $\pm$ 0·9, $n = 40$) in 30 min followed by a fall to 95 mV ($\pm$ 2·3, $n = 30$) in 1 hr. An increase of Ca^{2+} to 2·4 mM produced a maximum of 116 mV ($\pm$ 0·8, $n = 60$) reached in 1 hr, followed by a decline to 103 mV ($\pm$ 0·9, $n = 60$) in a further hour. The resting potential reached in 1 hr was found to be linearly related to the logarithm of the external Ca^{2+} concentration over the range 0·01–2·4 mM ($r = 0·97$, $n = 13$). An e-fold increase in Ca^{2+} caused a 4 mV increase in resting potential.

The muscles were replaced in Ringer's solution after 2 hr or when twitching became too violent. The presence of Ca^{2+} delayed the onset of the twitching which was associated with the decline of the hyperpolarization. Replacing the muscles in Ringer's solution resulted in a return to normal values of resting potential within 1 hr and thereafter the membrane potential remained stable for a further 2 hr.

Recent workers (Frankenhauser & Hodgkin, 1956; Huxley, 1959) have studied the role of Ca^{2+} in determining the selective permeability of the squid axon membrane particularly during activity. The present experiments indicate that in conditions where the external K^+ is very low the

selective permeability of the muscle membrane in the resting state is dependent to a marked degree on the Ca^{2+} concentration in the medium.

REFERENCES

BÜLBRING, E., HOLMAN, M. & LÜLLMANN, H. (1956). *J. Physiol.* **133**, 101–117.
DRAPER, M. H., FRIEBEL, H. & KARZEL, K. (1959). *J. Physiol.* **148**, 61–62*P*.
FRANKENHAEUSER, B. & HODGKIN, A. L. (1957). *J. Physiol.* **137**, 217–244.
HUXLEY, A. F. (1959). *Ann. N.Y. Acad. Sci.* **81**, 221–246.
WOODBURY, J. W. (1958). *Exp. Cell Res.* Suppl. **5**, 547–539.

Structure of acetylcholine receptors in intestinal smooth muscle.

By H. O. SCHILD. *Department of Pharmacology, University College London*

Evidence is presented suggesting that acetylcholine (ACh) receptors in guinea-pig ileum possess two reactive sites *A* and *B*. Site *A* reacts with the quaternary ammonium group of ACh and related compounds but fails to react with undissociated amines. Thus, at pH 9 the contractile effects of a weak base such as pilocarpine were abolished, whilst those of a strong base such as ACh were maintained.

In order to identify site *B*, the effects of ACh and tetramethylammonium (TMA) were tested after pre-treating the ileum preparation with various reagents. Any differential depression of ACh effects would indicate inactivation of site *B*, since TMA represents the cationic head of ACh which reacts only with site *A*. (The contractile action of TMA on ileum is mainly muscarinic since it is not abolished by hexamethonium.) Treatment of the ileum for 10 min with 8×10^{-5} polyethyleneimine—a polybase kindly provided by Professor Bergel—produced a marked differential depression of ACh, which persisted indefinitely. Polyethyleneimine produced these effects at pH 7 but not at pH 3·9. This may signify that site *B* contains an imidazole group, known to dissociate within this range. Differential depression of ACh also occurred after pre-treatment of the ileum with diazotized sulphanilic acid. This reagent probably reacts with imidazole groups. These findings suggest that the structure of the muscarinic ACh receptors in the ileum may be similar in some respects to the active surface of cholinesterase as revealed by the studies of Wilson & Bergmann (1950).

REFERENCE

WILSON, I. B. & BERGMANN, F. (1950). *J. biol. Chem.* **186**, 683.

Defence reactions from the amygdala and the stria terminalis.

By A. W. ZBROZYNA*. *National Institute for Medical Research, Mill Hill, London, N.W. 7*

Stimulation in the amygdaloid nuclei produces a defence reaction similar to that described by Hess on stimulation of the hypothalamus (Kaada, Andersen & Jansen, 1948; Magnus & Lammers, 1956; Shealy & Peele, 1957; De Molina & Hunsperger, 1959).

In the present study the defence area in the amygdala was mapped in conscious cats with stereotactically implanted electrodes; it was located in the basal nuclei. Stimulation of this area in anaesthetized cats did not result in muscle vasodilatation, but in conscious cats an atropine-sensitive vasodilatation was registered by the venous temperature method (Abrahams, 1959). Thus the defence reaction elicited from the amygdala, like that from the hypothalamus (Abrahams & Hilton, 1958), is associated with active muscle vasodilatation.

The resemblance of the defence reactions produced from the hypothalamus and amygdala strongly supports the suggestion that the amygdala produces this reaction through activation of the diencephalic centre. Anatomical studies led to the belief that the stria terminalis is the main pathway conducting from the amygdala to the hypothalamus (Johnston, 1923; Fox, 1943), and Gloor's (1955) finding of evoked potentials in the hypothalamus on stimulation of the amygdala appeared to support this view.

Recently, De Molina & Hunsperger (1959) found that stimulation of the stria terminalis produced a defence reaction. They therefore suggested that the stria connects the amygdala with the hypothalamic centre for the defence reaction. In the present study it was confirmed that stimulation of the stria produces the defence reaction. It was also found to produce the atropine-sensitive muscle vasodilatation. Nevertheless, the stria terminalis is not the efferent pathway from the amygdala, because lesions placed in the stria, either on one or on both sides, left the effect of stimulation in the amygdala unchanged. This finding raised the question whether the stria is not in fact conducting toward the defence area in the amygdala. Accordingly, an assembly of three electrodes was implanted along the course of the stria on one side, a lesion was made at the tip of the middle electrode and its effect was tested by stimulation via electrodes on each side of the lesion. The defence reaction was no longer obtainable from the electrode anterior to the lesion (on the hypothalamic side), while from the posterior electrode (on the amygdaloid side) a full defence reaction was obtained. These results show that the stria terminalis is not a pathway for the defence reaction from the amygdala to the hypothalamus but

* Rockefeller Fellow.

that it is an afferent route activating the amygdala. The pathway from the amygdala to the hypothalamic centre for the defence reaction is still unknown.

REFERENCES

ABRAHAMS, V. C. (1959). *J. Physiol.* **145**, 20*P*.
ABRAHAMS, V. C. & HILTON, S. M. (1958). *J. Physiol.* **140**, 16–17*P*.
FOX, C. A. (1943). *J. comp. Neurol.* **79**, 277.
GLOOR, P. (1955). *Electroenceph. clin. Neurophysiol.* **7**, 223.
JOHNSTON, J. B. (1923). *J. comp. Neurol.* **35**, 337.
KAADA, B. R., ANDERSEN, P. & JANSEN, J. (1948). *Neurology*, **48**, 454.
MAGNUS, O. & LAMMERS, H. J. (1956). *Folia psychiat. neerl.* **59**, 555.
DE MOLINA, A. F. & HUNSPERGER, R. W. (1959). *J. Physiol.* **145**, 251.
SHEALY, C. N. & PEELE, T. L. (1957). *J. Neurophysiol.* **20**, 125.

Some observations on the morphology of the intrafusal muscle fibre. By D. BARKER and J. L. GIDUMAL. *Department of Zoology, University of Hong Kong*

The intrafusal muscle fibres of the cat are of three sizes, large, medium, and small. In Zenker-fixed paraffin sections the diameters of the large fibres usually fall within the range of 15–25 μ, the medium fibres 10–15 μ, and the small 4–9 μ. The large fibres are the longest in the spindle and possess the characteristic myotube and nuclear-bag regions in the equatorial region; we have never observed them to divide. The medium fibres possess nuclear bags or nuclear chains; they may or may not divide in their course through the spindle. The small fibres lack nuclear bags but usually possess a nuclear chain in the equatorial region; they are the shortest fibres present and always divide. In cross-section the large and medium fibres exhibit Krüger's (1952) fibrillar pattern throughout most of their course, while the small fibres are predominantly areal.

The division of a medium or small intrafusal muscle fibre is quite distinct from the condition where one fibre tapers off alongside another. A fibre about to divide may be seen in transverse section to lose its circular contour and become first oval and then hour-glass in shape before finally splitting into two, the conversion taking place over a distance of 60–90 μ. Tapering occurs over much longer distances, usually 300–500 μ. The divided condition of intrafusal muscle fibres has been observed in spindles from rectus femoris, tenuissimus, teres minor, and intercostal muscles of cat; from pes interosseus III of hedgehog; and from extensor digitorum longus IV of frog.

Graphic reconstructions from serial transverse sections of five cat spindles (four single; one tandem) show that the products of medium and small-fibre division usually reunite, and that this may either result in the original 'parent' fibre being reformed, or in two 'parent' fibres sharing one 'daughter' fibre as a link between them. Fibre division usually

occurs in the proximal pole followed by reuniting in the distal pole so that the number of muscle fibres in the equatorial region is greater than at the poles, sometimes almost double. Both division and re-uniting may also occur within the equatorial region, though always towards either end of the capsule. The single spindles studied in the cat consisted of four to six muscle fibres, and division and reuniting occurred two to three times in each spindle.

This investigation is supported by research grant B-1806 from the National Institute of Neurological Diseases and Blindness, U.S. Public Health Service.

REFERENCE

KRÜGER, P. (1952). *Tetanus und Tonus der quergestreiften Skelettmuskeln der Wirbeltiere und des Menschen*. Leipzig: Akademische Verlagsgesellschaft.

Ionic movements in intestinal smooth muscle. By P. J. GOODFORD and K. HERMANSEN. *Department of Pharmacology, University of Oxford*

Forty-five pieces of guinea-pig taenia coli muscle were dissected by a standardized procedure, and immersed in modified Krebs's solution (136 mM-Na; 5·9 mM-K; 2·5 mM-Ca; 2 mM-Mg; 130 mM-Cl; 15 mM-HCO_3; 2·2 mM-phosphate; 11·5 mM-dextrose; 97 % O_2 + 3 % CO_2; pH 7·3; 35° C) with a tension of 2 g. The inulin space was determined on a further thirty-six pieces. A steady state was reached in which the ratios:

$$\frac{\text{wet wt.}}{\text{fresh wt.}} = 1{\cdot}02, \quad \frac{\text{dry wt.}}{\text{fresh wt.}} = 0{\cdot}191,$$

$$\frac{\text{Na (mM)}}{\text{kg wet wt.}} = 100, \quad \frac{\text{K (mM)}}{\text{kg wet wt.}} = 68, \quad \text{Inulin space} = 33\,\%,$$

did not change over a period of 6 hr. The ratio of cell volume to cell surface area V/A was 1·25 μ when determined histologically. Other pieces of taenia were transferred during the experimental period to a solution which contained either ^{24}Na or ^{42}K; they were removed after different times, dried, ashed, and their specific activities were compared with that of the solution. The specific activity of the sodium increased very rapidly ($t_{\frac{1}{2}} \doteqdot 1$ min) to beyond 90 % of that of the solution, and then continued to increase very slowly. The specific activity of the potassium rose more slowly, and appeared to show two components ($t_{\frac{1}{2}} = 50$ min and 8 hr).

From these observations it may be calculated that 55 % of the sodium was intracellular at a concentration of 80 mM/kg, and 97 % of the potassium at 100 mM/kg. An apparent hyperosmotic concentration of Na + K was also found by Daniel (1958). The potassium equilibrium potential would then be about −70 mV and the sodium equilibrium potential about 15 mV. Holman (1958) observed a mean resting potential of 51 mV, with

some values rising to 70 mV, and a mean spike amplitude of 59 mV. It is difficult to interpret our results on the basis of the Goldman equation.

REFERENCES

DANIEL, E. E. (1958). *Canad. J. Biochem. Physiol.* **36**, 805–818.
HOLMAN, M. E. (1958). *J. Physiol.* **141**, 464–488.

The metabolic basis for the inhibitory action of adrenaline on intestinal smooth muscle. By J. AXELSSON and EDITH BÜLBRING.
Department of Pharmacology, University of Oxford

Isolated guinea-pig taenia coli preparations were used for recording electrical and mechanical changes. Adrenaline relaxes this muscle because it stops spontaneous spike discharge. It renders the tissue inexcitable and it causes hyperpolarization (Bülbring, 1954; Burnstock, 1958). Axelsson, Bueding & Bülbring (1959) showed that adrenaline caused an increase in phosphorylase activity, and that this effect coincided with the electrical changes. When the metabolic action was prevented by prolonged exposure to glucose-free medium the effect of adrenaline was abolished. It was concluded that the activation of phosphorylase increased the rate of energy supply and led to an acceleration of the sodium pump.

When the metabolic rate was increased by raising the temperature from 28 to 38° C, an immediate cessation of spontaneous spike discharge and hyperpolarization was observed. This initial stabilization of the membrane lasted for several minutes before the new activity at higher temperature became established.

Recently Bülbring & Goodford (unpublished results) found that when adrenaline relaxed the muscle, the rate of loss of ^{24}Na was increased and the rate of ^{24}Na uptake was diminished.

The electrical changes normally produced by adrenaline were not seen when the sodium of the medium was substituted by lithium. If in taenia coli lithium can only be extruded at a very slow rate, as shown for frog skeletal muscle by Keynes & Swan (1959), this may explain why adrenaline fails to produce its usual effect.

The results obtained are consistent with the view that adrenaline causes its inhibitory effect on intestinal muscle by stimulating one reaction in a chain of metabolic cycles supplying energy for active mechanisms which stabilize the smooth muscle membrane.

REFERENCES

AXELSSON, J., BUEDING, E. & BÜLBRING, E. (1959). *J. Physiol.* **148**, 62–63*P*.
BÜLBRING, E. (1954). *J. Physiol.* **125**, 302–315.
BURNSTOCK, G. (1958). *J. Physiol.* **143**, 183–194.
KEYNES, R. D. & SWAN, R. C. (1959). *J. Physiol.* **147**, 626–638.

Histidine decarboxylase activity in a malignant rat hepatoma. By D. MACKAY, P. B. MARSHALL and J. F. RILEY. *Department of Pharmacology and Therapeutics, Queen's College, Dundee, University of St Andrews*

According to Kahlson (1960) the adaptive enzyme, histidine decarboxylase, undergoes dramatic increase in rat liver during active normal growth, as in the liver of the developing foetus and in adult liver regenerating after partial hepatectomy. The increased enzymatic activity in female rats is indicated by a rise in free histamine in the urine.

We therefore tested the histidine decarboxylase activity of yet another rapidly growing type of liver tissue, a transplantable hepatoma (F-Hep)* which grows as a subcutaneous transplant in rats of the August strain.

In female rats bearing a subcutaneously implanted hepatoma, the urinary histamine rose steeply from the normal level of 30–40 μg per rat per 24 hr to values in excess of 1 mg per rat per day. On removal of the tumour, the histamine excretion at once fell to the normal level. Portions of tumour were implanted into the controls whose urinary histamine values now rose.

There is very little histidine decarboxylase activity in normal adult rat liver. High activity was found in homogenates of hepatoma tissue (approximately one-eighth the activity of guinea-pig kidney) and this was increased tenfold on the addition of pyridoxal-5′-phosphate.

REFERENCE

KAHLSON, G. (1960). *Lancet*, **278**, 67–71.

Intrahepatic vascular pathways and the hepatic nerves. By W. H. H. ANDREWS and I. DEL RIO LOZANO. *Department of Pharmacology, St Mary's Hospital Medical School, London, W. 2*

Cannulae were inserted into the hepato-duodenal artery and a mesenteric vein of anaesthetized dogs, cats and rabbits. Acetylcholine, noradrenaline and other substances were injected into the hepatic arterial and portal venous circulations and variations of general arterial pressure were used as indications of the amount of drug passing unchanged through the liver. These drugs affect the liver vessels and presumably influence the results. There was variation from animal to animal, but it did appear that the amount of drug destroyed by the liver could be changed by inhibiting the action of the hepatic nerves in all but three animals. Changes of tone in the hepatic artery or portal vein do not, by themselves, materially affect general arterial pressure.

* Obtained through the kindness of Professor A. Haddow and Dr K. S. Kirby, Institute of Cancer Research, Royal Cancer Hospital, London.

The effectiveness of hepatic arterial and portal venous injection were compared and the results expressed as a ratio. For acetylcholine the ratio was usually between 5 and 20:1; more drug was needed to produce a given response when injected into the portal vein than when into the artery. In two cats and one dog the ratio was between 1:1 and 3:1 and remained so throughout the experiment. Administration of acetylcholine via the femoral vein was from five to fifty times as effective as hepatic arterial injection. In six dogs the nerves which accompany the hepatic artery were cut. In one the responses to acetylcholine were unchanged; in two, both hepatic arterial and portal venous injection had a bigger effect, and in the remaining three, hepatic arterial but not portal venous injection produced a bigger response. Hexamethonium bromide had a different effect and tended to reduce the ratio to 1:1, whether the nerves had been previously cut or not. In the one dog in which it was tried, bretylium changed the ratio from 5:1 to 1:2. In cats section of nerves had a variable effect, but hexamethonium acted as in dogs.

With adrenaline and noradrenaline the ratio of effectiveness between hepatic arterial and portal venous injection was less, though it followed the same pattern as acetylcholine injection. In rabbits, however, the ratio was between 5 and 15:1. After the administration of choline 2:6 xylyl ether bromide the ratio was considerably reduced.

Autonomic nerves therefore appear to regulate hepatic vascular pathways, but another method will have to be used to obtain more precise information. Nerve section has less effect than have inhibitors of nerve function, and the direction of change is not the same. No nerves other than those accompanying the hepatic artery have been found with a direct action on the liver blood flow (Andrews, Hecker & Maegraith, 1958) and the possibility of intrahepatic nerve relays (cf. Burn & Rand, 1960) may have to be considered.

The work was aided by a grant from the Medical Research Council.

REFERENCES

Andrews, W. H. H., Hecker, R. & Maegraith, B. G. (1958). *Ann. trop. Med. Parasit.* **52**, 500–507.

Burn, J. H. & Rand, M. J. (1960). *Brit. J. Pharmacol.* **15**, 55–66.

Sheep heart; low resistance of intercalated disks to the movement of ^{42}K. By S. Weidmann. *Universität Bern, Hallerianum, Physiologisches Institut, Switzerland*

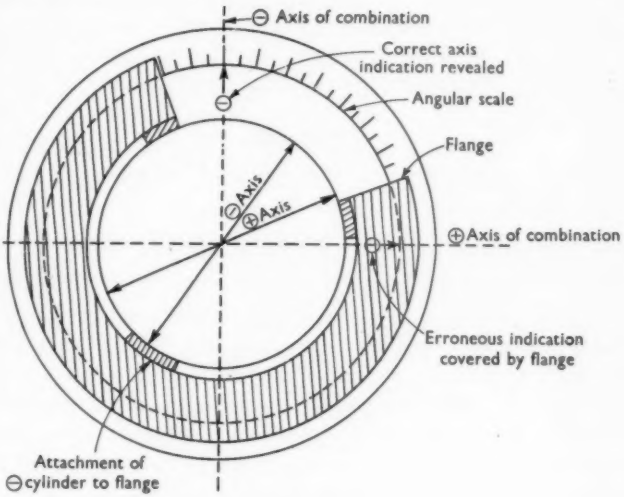
PROCEEDINGS OF
THE PHYSIOLOGICAL SOCIETY

CAMBRIDGE MEETING

9 *July* 1960

An instrument for measuring refractive errors. By H. M. F. ASHER. *Department of Physiology, The Medical School, University of Birmingham*

The instrument demonstrated is a prototype developed from the laboratory device previously demonstrated (Asher, 1956), and is intended for clinical use.



Cross cylinder unit

The chief modification concerns the Cross-Cylinder Unit. The relative rotation of the +3D and −3D cylinders may now take place in either direction from the zero (axes parallel) position. Suppose that when parallel the axes are at an angle of 45° to the vertical. Let the cylinders rotate so that the positive axis approaches the vertical and the negative the horizontal direction. The combination is now equivalent to a vertical + and a horizontal − cylinder whose strengths increase with the angle of

rotation, and reach a maximum value of + 3D and − 3D when the cylinder axes are vertical and horizontal respectively. It can be shown that this combination is equivalent to a − 6D cylinder axis horizontal combined with a + 3D sphere. If, however, starting from the zero position, the rotation takes place in the reverse sense, then the combination becomes equivalent to a vertical negative and a horizontal positive cylinder, whose power increases to a limit of − 3D and + 3D. This combination is in turn equivalent to a − 6D cylinder axis vertical and a + 3D sphere.

Two arrows are marked on the framework of the Cross-Cylinder Unit (see figure), and according to the direction of rotation of the cylinders, either one arrow or the other will represent the minus axis of the combination. A flange from which a 90° sector has been removed is rigidly fixed to the minus cylinder so as to rotate with it. Only the arrow currently representing the direction of the minus axis is visible through the missing sector. The Unit as a whole may be rotated, and the axis of the patient's cylindrical defect (which is always recorded as minus) is read from the arrow against an angular scale.

The instrument has been designed and constructed by Research Engineers Ltd., Northampton Grove, Canonbury, London, N. 1.

REFERENCE

Asher, H. M. F. (1956). *J. Physiol.* **134**, 4*P*.

Rhythmic properties of insect fibrillar muscle. By K. E. Machin and J. W. S. Pringle. *Department of Zoology, University of Cambridge*

The rhythmic properties of insect fibrillar muscle can be demonstrated simply by suspending a weight in air from one end of the muscle. The demonstration shows the independence of electrical and mechanical phenomena and the dependence of the frequency of contraction on the inertia of the load (Machin & Pringle, 1959). Measurements made with more elaborate (not transportable) apparatus (Machin & Pringle, 1960) have shown that the development of tension and the generation of oscillations are distinct properties of this muscle which can, under certain circumstances, be manifested independently.

REFERENCES

Machin, K. E. & Pringle, J. W. S. (1959). *Proc. Roy. Soc.* B, **151**, 204.
Machin, K. E. & Pringle, J. W. S. (1960). *Proc. Roy. Soc.* B, **152**, 311.

Measurement of temperature changes during the discharge of *Electrophorus electricus*. By X. M. J. AUBERT and R. D. KEYNES. *Physiological Laboratory, University of Cambridge*

The isolation of synaptic vesicles from the central nervous system. By E. G. GRAY and V. P. WHITTAKER. *Department of Anatomy, University College London, and Biochemistry Department, Agricultural Research Council Institute of Animal Physiology, Babraham, Cambridge*

Previous work (Hebb & Whittaker, 1958; Whittaker, 1959) showed that it is possible to isolate from sucrose homogenates of guinea-pig forebrain a subcellular particulate fraction distinct from nuclei, mitochondria and microsomes, and containing most of the bound acetylcholine and hydroxytryptamine of the tissue. This fraction (*B* layer of P_2; Whittaker, 1959) has an equilibrium density in a sucrose density gradient intermediate between that of 0·8 and 1·0 M sucrose. After fixing with osmium tetroxide, embedding in methacrylate and examination with the electron microscope (observations by K. M. Smith & G. J. Hills, quoted by Whittaker, 1959) the fraction was seen to consist of particles with diameters similar to those of synaptic vesicles (about 500 Å).

The various fractions (P_1, P_2, P_3) have now been studied in the electron microscope by fixation with osmium tetroxide containing sucrose, and dehydration without resuspension. This procedure has the advantage of preserving delicate membranes. The material was subsequently stained with phosphotungstic acid and embedded in araldite, a method that has proved particularly valuable in the study of central nervous synapses (Gray, 1959). In the *B* fraction the smallest particles are now clearly seen to be synaptic vesicles (Fig. 1) that are enveloped within membranes forming larger particles. These are identified as pinched-off nerve endings because they contain (*a*) the characteristic synaptic vesicles and (*b*) mitochondria, and (*c*) because the thickened region of the post-synaptic membrane sometimes remains attached as a marker. Possibly the thickening remains attached to the majority of endings, but only occasionally appears in the plane of section as would be expected. Thus the complete structure is identical with the endings seen in sections of cerebral cortex (Gray, 1959). The ending is apparently torn off during homogenization and survives centrifugation intact. Similarly, in sections of damaged cerebral cortex, the endings survive mechanical disruption and the post-synaptic thickening remains attached, although the rest of the post-synaptic process has disintegrated (Gray, 1959). A number of small particles (*x*) are also present in the

B fraction, and until they have been identified conclusions must be tentative.

Fraction P_1 is seen to contain nuclei, blood vessel fragments and myelin; P_3 contains the microsomes; P_2 has three subfractions. *A* contains disrupted myelin and *C* the mitochondria. It is only in the *B*

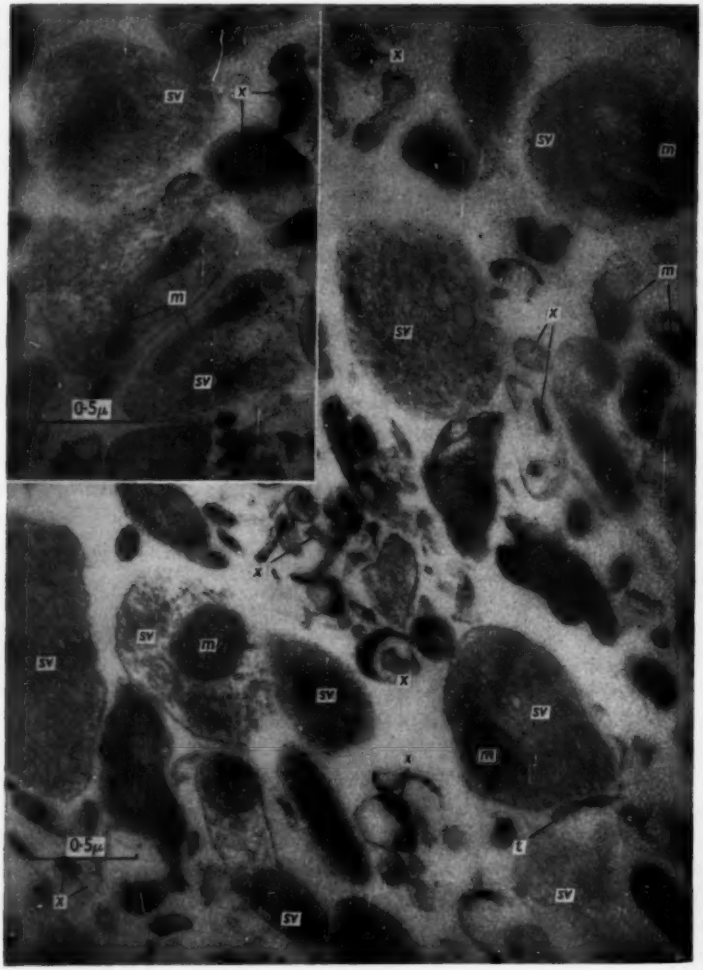


Fig. 1. Electron micrographs of fraction *B* from guinea-pig forebrain. *m*, mitochondria, *sv*, synaptic vesicles; *t*, thickening of post-synaptic membrane, *x*, unidentified particles.

layer that the endings, tightly packed with synaptic vesicles, are concentrated, and here also substances to which a transmitter action has been assigned are present. Thus a relationship between transmitter substance and synaptic vesicles has been established, although further experiments are necessary to establish that the transmitter is confined within the vesicles and not merely within the presynaptic membrane. Furthermore, by this method it should be possible to obtain samples of intact nerve endings from localized regions of the central nervous system and so detect and analyse their transmitter substances by direct methods.

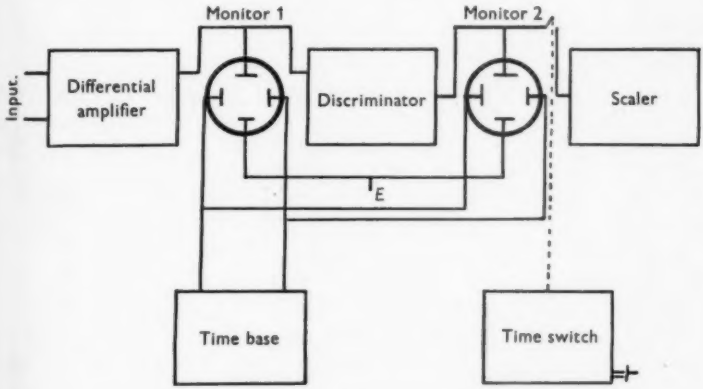
REFERENCES

GRAY, E. G. (1959). *J. Anat., Lond.*, **93**, 420.
HEBB, C. O. WHITTAKER, V. P. (1958). *J. Physiol.* **142**, 187.
WHITTAKER, V. P. (1959). *Biochem. J.* **72**, 694.

Apparatus for recording the heart rate in conscious animals.

By E. M. GLASER, J. P. GRIFFIN and D. KNIGHT. *Department of Physiology, The London Hospital Medical College, London, E.* 1

The apparatus can be used to provide in quick succession any number of accurate counts of the heart rate from conscious animals, including small animals with heart rates of 10 or more beats/sec.



The electrical impulse of the heart is picked up from small indwelling platinum electrodes which can be inserted into the skin under light anaesthesia and which cause no subsequent discomfort. The amplifier has a differential input, and it is resistance-capacity coupled, with an amplification of 15×10^4 and a frequency response of 10 c/s to 10 kc/s. The final stage is a compressor stage giving 'single-ended' output, and this is

monitored by a 1 in. (2·5 cm) cathode-ray tube. It is also connected to a discriminator which has a variable adjustment, allowing selection of the *R* spike of the e.c.g. and the discriminated signal is then monitored by another 1 in. cathode-ray tube. Thus a direct comparison can be made between the amplified signal from the skin electrodes and the discriminated signal, with elimination of all interference, especially from muscles.

Counting is done by a decade scaler into which the discriminated signal is fed via a time switch. The scaler consists of three Mullard 'stepping tubes' preceded by a fixed filter. The maximum count rate of the scaler is 4000 pulses/sec. Monitoring of the scaler during each count gives a further visual check.

The time switch consists of a cathode coupled 'flip-flop' circuit which allows counting over 15 sec with an error of $\pm 5\%$. Fully automatic counting with great accuracy over varying lengths of time can be obtained by inserting a time switch with crystal control. It is also possible to control the time of each count by an overriding manual switch.

A multiple switch on the input side allows quick changes from one animal to another, if necessary. Permanent records of the count can be obtained by feeding the signal into an oscilloscope with a camera or into a direct-writing device. The apparatus can be used for counting the heart rate in man by using ordinary skin electrodes, and it can be converted for almost any physiological experiment in which electrical impulses have to be counted over given periods of time.

A method of applying temperature compensation to capacitance transducer heads. By G. W. CAMBRIDGE and J. HAINES. *Pharmacology Laboratory, John Wyeth and Brother Limited, Havant, Hants*

By means of the quartz crystal-controlled transducer system previously described (Cambridge & Haines, 1959) it is possible to check and adjust for any internal drift arising in the system itself when considered without the transducer elements. A drift of less than 10 mV on a 20 V output is normal after thermal equilibrium has been reached. Drift caused by a change in temperature affecting the transducing element may then become very much greater than the internal drift. This applies, for example, to capacitance-type pressure transducers which may be exposed to varying environmental temperature. This drift is the result of mechanical forces which alter the relative disposition of the plates of the capacitor.

A method of temperature compensation will be demonstrated which reduces this drift to negligible proportions. It is based on the use of negative temperature-coefficient capacitors in parallel with the capacitance plates, the values being calculated to produce an over-all zero temperature

coefficient. This type of compensation can also be applied to the inductance-change transducer head which can be used with this system.

REFERENCE

CAMBRIDGE, G. W. & HAINES, J. (1959). *J. Physiol.* **149**, 2–3*P*.

Eructation and rumen movements in sheep. By C. S. W. REID.
Physiological Laboratory, University of Cambridge

A sequence of contractions of the reticulum and rumen, recurring at intervals of 30–180 sec, has been described in sheep and cattle (Schalk & Amadon, 1928; Phillipson, 1939). This consists normally of a diphasic contraction of the reticulum, followed by a variable cycle of contractions of the rumen which successively involves its different structures. The nature of other rumen contractions, which may occur independently of those of the reticulum, is less well defined. Many of these contractions occur in a definite relationship to eructation; this is not constant, nor are the contractions essential for eructation to occur.

With partial exteriorizations of the reticulum and different regions of the rumen (Titchen, 1958), it has proved possible to define more clearly the sequence of contractions with which eructation may be associated. Direct observation, cine-photography and the technique of recording previously described (Reid & Titchen, 1959) have been used for this purpose.

Weiss (1953) deduced from pressure records that rumen contractions associated with eructation successively involved first the more posterior regions of the dorsal sac and later the anterior regions of the dorsal rumen. The present evidence indicates that the sequence of events varies considerably, but most commonly the first contraction seen is one of the posterior ventral blind sac of the rumen. The onset of this contraction precedes by 1–7 sec one of the dorsal rumen. This contraction of the dorsal rumen, in contrast to those associated with reticulum contractions, appears to involve all parts simultaneously and is also accompanied by a weak contraction of the ventral sac of the rumen. A contraction of abdominal muscles, followed by eructation, may occur during the contraction of the dorsal sac of the rumen. The whole sequence finishes with a strong contraction of the main ventral sac of the rumen.

The dorsal rumen contractions associated with contractions of the reticulum occur first in the anterior region and then after 0·2–2·0 sec in the more posterior region. These contractions are stronger and of longer duration than those which occur independently.

REFERENCES

PHILLIPSON, A. T. (1939). *Quart. J. exp. Physiol.* **29**, 395–415.
REID, C. S. W. & TITCHEN, D. A. (1959). *J. Physiol.* **149**, 14–15*P*.
SCHALK, A. F. & AMADON, R. S. (1928). *Bull. N. Dakota Exp. Sta.* **216**.
TITCHEN, D. A. (1958). *J. Physiol.* **143**, 35*P*.
WEISS, K. E. (1953). *Onderstepoort J. Vet. Res.* **26**, 251–283.

Myelinating and non-myelinating nerve fibres during development: a new component of the endoplasmic reticulum of Schwann cells. By J. D. ROBERTSON. *Department of Anatomy, University College London*

Developing mouse sciatic nerves were studied by electron microscopy, by using serial sectioning techniques, OsO_4 and $KMnO_4$ fixation and various staining procedures. Selected micrographs illustrate several new findings.

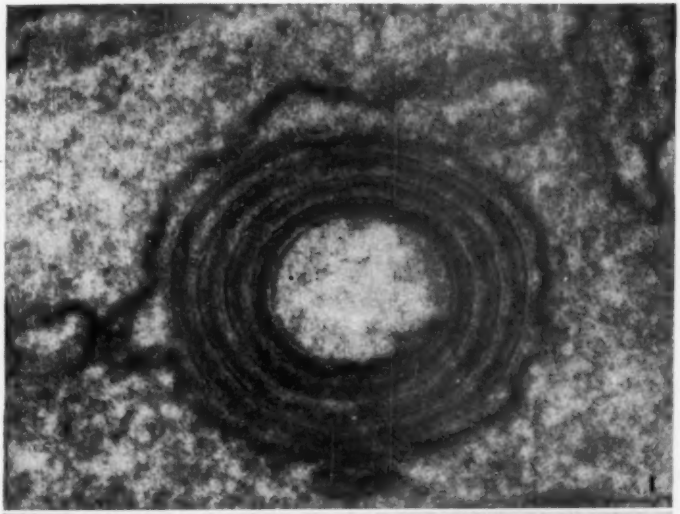
First, serial transverse sections completely through several nodes of Ranvier have shown that the direction of the myelin spiral from one internodal segment to the next is sometimes the same and sometimes reversed.

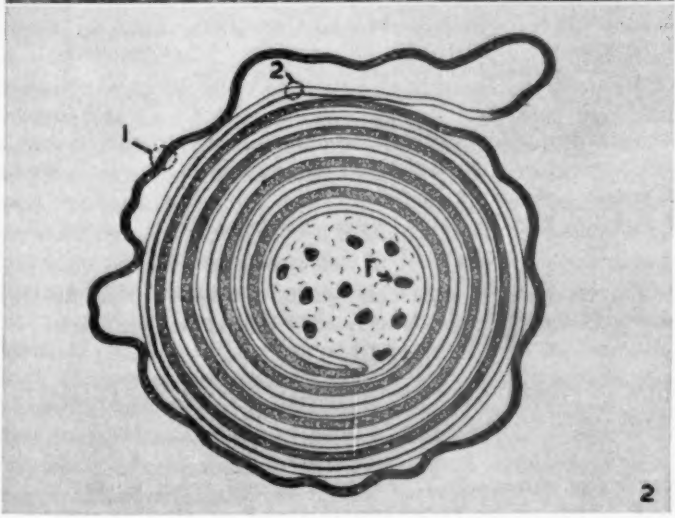
Non-myelinating fibres during early development (Robertson, 1960) resemble olfactory nerve fibres (Gasser, 1956) in that bundles of large numbers of axons are seen enveloped by one or more Schwann cells. The axons in OsO_4 fixed preparations contain numerous transected neurofilaments and the Schwann cells have relatively abundant granular endoplasmic reticulum (ER). The neurofilaments measure from < 100 Å in diameter to $\sim$ 200 Å. The smaller ones appear solid and the larger ones have cores of lower density.

There is a marked variation in the diameters of the axons in a given plane of section. These range from $\sim$ 750–1000 Å up to more than a micron in diameter. Serial reconstructions have established that the smallest profiles are constrictions along the longitudinal axis of large axons locally reducing their diameter by a factor of about ten. These constrictions are also common in myelinating fibres. This is shown in serial reconstructions. It may be that the constrictions are instrumental in the formation of myelin by inducing a spiral growth of the inner lip of the Schwann cell next to the axon.

During the formation of myelin, after several spiral lamellae have been laid down, the outer nucleated layer of the Schwann cell becomes enlarged and packed with granular endoplasmic reticulum and moderate numbers of a peculiar spiral organelle for which the term 'growth spiral' is proposed. A transection of a growth spiral after $KMnO_4$ fixation is shown in Fig. 1. This is interpreted diagrammatically in Fig. 2. These structures are $\sim 0{\cdot}3$–$0{\cdot}4\,\mu$ in diameter and several microns long. They are disposed

parallel to the long axis of the nerve. Each consists essentially of a flattened sac of the ER wrapped in a perfect simple spiral about a central core of cytoplasmic matrix containing ribosomes ('*r*', Fig. 2). The spiral sac is bounded by a unit membrane that is differentiated in two ways. The outer component of each loop (1, Fig. 2), while measuring ~ 75 Å in thickness,





appears dense and granular and does not show the characteristic three strata of the unit membrane. The inner membrane into which the outer one changes gradually (2, Fig. 2) does show the three characteristic strata, but the cytoplasmic dense layer is unusually prominent and thickened slightly. The content of the spirally wound sac bounded by the differentiated membranes is unremarkable and in some places obliterated by contact of the membranes. There is a thin ($\sim$ 100 Å) layer of cytoplasmic matrix enclosed between the loops of the sac. This appears more dense after uranyl acetate or lead hydroxide staining and may thus contain closely packed ribosomes. The outermost membrane surface has more discrete and obvious attached ribosomes. The entire structure is cigar-shaped, tapering at its ends by a reduction in the number of loops of the spiral sac. The sac has been traced in serial sections into continuity with ordinary cisternae of the granular ER. The core opens directly into the cytoplasmic matrix without a constriction. This structure may represent a centre of synthesis of ER membranes. The spiral structure may result from a higher rate of growth of the outer membrane bounding the sac.

One might draw an analogy between the growth spiral and a spirally wrapped bimetallic sheet, the outer member of which expands more rapidly on heating. The outer membrane of the growth spiral would expand in an analogous way if it were the seat of relatively intense synthetic activity and that there is some difference here is indicated by the different fixation characteristics.

REFERENCES

GASSER, H. S. (1956). *J. gen. Physiol.* **39**, 473–496.
ROBERTSON, J. D. (1960). *Fourth International Conference on Electron Microscopy, Berlin*, Band II: Berlin: Springer Verlag.

A micromanipulator for the penetration of Protozoa and biological tissues with saline-filled electrodes. By M. S. BINGLEY. *Department of Zoology, University of London King's College, London, W.C.* 2 and E. SAUNDERS-SINGER, *London Street, Reading*

A mains-driven biological DC amplifier. By M. S. BINGLEY. *Department of Zoology, University of London King's College, London, W.C* 2

A model of auditory ossicles (film). By B. H. C. MATTHEWS. *Physiological Laboratory, University of Cambridge*

Axo-dendritic synapses of the tectospinal tract in the chicken. By P. GLEES. *University Laboratory of Physiology, Oxford*

Some tricks with correlation. By P. E. K. DONALDSON and H. B. BARLOW. *Physiological Laboratory, University of Cambridge*

A versatile console for electrophysiology. By P. E. K. DONALDSON. *Physiological Laboratory, University of Cambridge*

Multi-channel pressure recording. By F. HOWARTH. *Department of Pharmacology, University of Cambridge*

A technique for reversible functional adrenalectomy in the living dog. By E. B. VERNEY. *Department of Pharmacology, University of Cambridge*

The effect of anticholinesterases on the action of acetylcholine on mammalian non-myelinated nerve fibres. By CHRISTINE J. ARMETT and J. M. RITCHIE. *Albert Einstein College of Medicine, New York* 61, *N.Y., U.S.A.*

Recent experiments on isolated desheathed nerves mounted in the sucrose-gap apparatus described by Stämpfli (1954) have shown that acetylcholine in a concentration of 3×10^{-4} g/ml. depolarizes the non-myelinated fibres in the rabbit's thoracic vagus trunk by more than 1 mV (Armett & Ritchie, 1960). The acetylcholine also reduces the spike height by about 30%, causes a characteristic enhancement of the spike after-positivity and slows conduction. These latter changes are probably a direct result of the depolarization because the first two of them can be reversed by hyperpolarizing the preparation with an external current. (The effect of hyperpolarization on conduction velocity has not been studied.) Carbachol (3×10^{-4} g/ml.) has an effect similar to that of acetylcholine.

Since it is well known that anticholinesterases *potentiate* the action of acetylcholine at various junctional sites, we were surprised to find that in the presence of eserine ($1-3 \times 10^{-5}$ g/ml.) the action of acetylcholine on the non-myelinated fibres was *reduced*. Thus, in a series of three successive tests on one nerve where two exposures to a concentration of acetylcholine 3×10^{-4} g/ml. in the absence of eserine caused reductions in spike height of 26 and 27%, respectively, a third exposure to the same amount of acetylcholine in the presence of eserine 3×10^{-5} g/ml. caused a reduction of only 9%; usually the effect of eserine was even more pronounced and the response to acetylcholine was sometimes completely abolished.

The action of acetylcholine was also much reduced or abolished by the anticholinesterases, DFP and prostigmine; it was similarly reduced by edrophonium which has some anticholinesterase activity. The

concentrations required however (10^{-4} – 10^{-3} g/ml.) are much higher than are required with eserine.

Two possible explanations for the effect of these anticholinesterases in blocking the action of acetylcholine are: first, that they allow accumulation of acetylcholine at some receptor site in such excessive amounts that the receptor becomes refractory to the acetylcholine; and secondly, that the anticholinesterases actually compete with acetylcholine at this site and thus block its action. Since in the untreated nerve a thirtyfold increase in the testing dose of acetylcholine (from 3×10^{-4} to 10^{-2} g/ml.) does not lead to a smaller reduction in the spike height, and since the action of carbachol (a drug which is not hydrolysed by cholinesterase) is also inhibited by the anticholinesterases used in the present experiment, the first possibility can be rejected and the second intriguing possibility remains.

REFERENCES

ARMETT, CHRISTINE J. & RITCHIE, J. M. (1960). *J. Physiol.* **152**, 141–158.
STÄMPFLI, R. (1954). *Experientia*, **10**, 508–509.

Changes in adrenal cortical function after pituitary stalk section in the rhesus monkey. By P. M. DANIEL*, O. E. PRATT and C. S. TREIP*. *Department of Neuropathology, Institute of Psychiatry, Maudsley Hospital, Denmark Hill, London, S.E.* 5

Cutting the pituitary stalk in rhesus monkeys causes extensive necrosis of the anterior lobe of the pituitary (Daniel, Prichard & Smith, 1959). The long-term effects of this procedure on adrenal cortical function have been assessed in nine rhesus monkeys by determining the plasma level of steroid (assumed to be mainly hydrocortisone) which gives a Porter-Silber (1950) reaction and by estimating the insulin tolerance. An impenetrable barrier was placed between the hypothalamus and the pituitary after the stalk had been cut. Mid-morning estimations of plasma steroid levels were made before operation and from 1 to 34 weeks afterwards. The solvent extraction and micropartition method used was based on that of Eik-Nes (1957) and the results expressed as 'hydrocortisone'. The pre-operative level was 24·8 μg/100 ml. (S.D. of mean $\pm$0·8 μg/100 ml.). It fell sharply after operation to a minimum value of about 25–30 % of the pre-operative levels after 15–20 days, but rose to 60–70 % of the pre-operative level after 50–100 days survival (Fig. 1).

Insulin tolerance tests carried out before and 1–5 months after operation showed increased sensitivity to insulin after operation, but the blood glucose rose in a normal way towards the initial level some 30–40 min

* Assisted by a grant from the Ford Foundation.

after the insulin dose. Knobil & Greep (1959) found no such rise in hypophysectomized monkeys unless cortisone or pituitary extract had been given shortly before the insulin tolerance test.

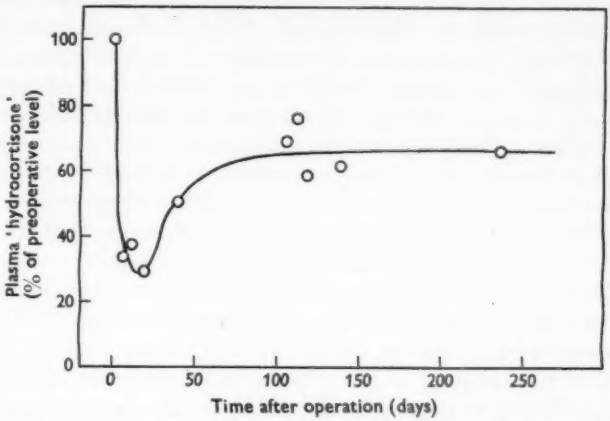


Fig. 1. Effect of pituitary stalk section on the 'hydrocortisone' (free steroid giving a Porter–Silber reaction) in the plasma of peripheral blood of nine rhesus monkeys.

Our findings suggest that there was a partial recovery of adrenal cortical steroid secretion some weeks after pituitary stalk section, despite a permanent interruption of the hypothalamohypophysial pathway.

REFERENCES

Daniel, P. M., Prichard, M. M. L. & Smith, B. (1959). *J. Physiol.* **146**, 2–3*P*.
Eik-Nes, K. (1957). *J. clin. Endocrin.* **17**, 502–511.
Knobil, E. & Greep, R. O. (1959). *Recent Progr. Hormone Res.* **15**, 1–58.
Porter, C. C. & Silber, R. H. (1950). *J. biol. Chem.* **185**, 201–207.

The distribution of light in an image formed in the cat's eye. By F. H. C. Marriott and Valerie B. Morris. *University Laboratory of Physiology, Oxford*

Temperature and the action of denervated sweat glands in the cat's foot pad. By D. P. C. Lloyd. *The Rockefeller Institute, New York, U.S.A.*

Experiments have been designed to investigate in controlled circumstances one facet of the problem of thermal sweating; namely, the action of the peripheral effector itself. Temperature has been varied over a physiological range avoiding unduly high and potentially or actually

injurious temperatures that might be expected to cause non-nervous secretion by damage (Randall, 1947).

Latency for sweat emergence in response to a test motor nerve stimulation varies linearly with duration of the rest period permitted following a maximal conditioning stimulation (Lloyd, 1959). On varying temperatures the linear relation is preserved, but the slope of the plot varies, latencies becoming shorter at elevated temperatures. The permitted rest period being fixed in duration, emergence latency varies linearly and inversely as the temperature. Reabsorption rate, as measured by recovery of impedance across the foot pad following a bout of maximal activity (Lloyd, 1960), varies as the temperature. In short, a rise in temperature enhances both secretory and reabsorptive activity of the sweat glands, the former necessarily in greater degree than the latter. This implies that the sweat glands during thermal sweating would yield per nerve impulse sweat in amount varying with local temperature. It does not necessarily imply that non-nervous thermal sweating occurs.

To test the possibility that sweat formation, perhaps in amount insufficient to constitute sweating, occurs during and as a direct consequence of warming, potential changes across the foot pad have been recorded and these compared with changes due to the action of injected pilocarpine. During sweat formation and consequent duct filling the maximal action potential in response to single motor volleys, as recorded, increases in magnitude due to lowering of in-series resistance. The change is large following pilocarpine injection; it is doubtfully present on warming. These results are inconclusive, but provide considerable evidence for supposing that non-nervous sweating, if indeed it occurs at all, is a negligible factor in the causation of increased sweat gland function at increased temperature.

REFERENCES

LLOYD, D. P. C. (1959). *Proc. nat. Acad. Sci., Wash.*, **45**, 405–409.
LLOYD, D. P. C. (1960). *J. gen. Physiol.* **43**, 713–722.
RANDALL, W. C. (1947). *Amer. J. Physiol.* **150**, 365–371.

The parts played by superficial and by deep temperature receptors in raising the metabolic rate of fat and thin men exposed to cold. By W. R. KEATINGE*. *Department of Experimental Medicine, Medical Research Council and University of Cambridge*

Both surface (Uprus, Gaylor & Carmichael, 1935; Spurr, Hutt & Horvath, 1957) and deep (Sherrington, 1923–4; Carlson, 1954) temperature receptors play a part in the metabolic response to cold, but their relative importance is uncertain. Since fat men can reduce their loss of

* Elmore Student, and Research Fellow of Pembroke College, Cambridge.

heat more than thin men in cold water, the metabolic response of fat and thin men to cold water has been measured both immediately after immersion when cutaneous stimulation was likely to be important, and after they had reached a steady state of heat exchange when deep temperature might play a greater part. First, ten fat and thin men were immersed motionless for 30 min in water at 15° C. In the first 10 min the metabolic rates of thin men were little higher than those of fat men, and there were considerable individual variations in metabolic rate not related to fatness. The metabolic rates of thin men only rose substantially above those of the fat men in the next 20 min when the thin men's rectal temperatures were falling rapidly.

Fat and thin men were then allowed to achieve temperature stability in water at various temperatures. Their metabolic rates always rose if the water was colder than 33–34° C, but those of thin men rose considerably more than those of fat men. The stable rectal temperatures of the thin men were lower in cold than warmer water, while those of fat men were not. It is concluded that cutaneous receptors play a large part in the metabolic response of fat and thin men to cold of short duration, and of fat men during longer exposure to cold, but that stimulation of deep temperature receptors enables thin men to match their metabolic rate to their relatively high heat loss during long exposures to cold.

REFERENCES

Carlson, L. D. (1954). *Proc. Soc. exp. Biol., N.Y.*, **85**, 303–305.
Sherrington, C. S. (1923–24). *J. Physiol.* **58**, 405–424.
Spurr, G. B., Hutt, B. K. & Horvath, S. M. (1957). *J. appl. Physiol.* **11**, 58–64.
Uprus, V., Gaylor, G. B. & Carmichael, E. A. (1935). *Brain*, **58**, 220–232.

The effects of intraventricular iproniazid on the rat. By L. Sinclair. *Department of Pharmacology, Guy's Hospital Medical School, London, S.E.* 1

A characteristic series of behavioural changes followed the injection of doses of 40–120 μg of iproniazid into the lateral ventricles of conscious rats by the technique of Lindsay, Robson & Sinclair (1959). In all animals there was an initial phase of excessive movements of the vibrissae and rapid rhythmic movements of the jaws. Following this, in most animals there was an increase of muscle tone and waxy flexibility of the limbs on the contralateral side. There were also clonic movements of the head, trunk and limbs, and in some animals, at the higher dose range, seizures occurred. The latter commenced on one side of the head, then involved the pinna and shoulder muscles and finally the trunk and limbs; they lasted for up to 15 sec. The total duration of the effects produced varied between 30 and 120 min.

Similar changes of behaviour were recorded after the intraventricular injection of similar doses of isoniazid, which is also a hydrazine derivative but which is not an amine oxidase inhibitor. They could be prevented by the previous intraperitoneal injection of pyridoxine (10 mg/kg) and γ-amino butyric acid (400 mg/kg) or the previous intraventricular administration of 60 μg of Pyridoxal.

Killam & Bain (1957) and Killam (1957) have shown that hydrazides increase seizure activity in the cat brain and also deplete it of γ-amino butyric acid by inhibiting the decarboxylation of L-glutamate. Dasgupta, Killam & Killam (1958) have reported that hydrazide induced hippocampal seizures in cats may be prevented by the pre-administration of γ-aminobutyric acid intraventricularly. It is suggested that the effects produced by iproniazid and isoniazid in the experiments reported here may be related to the cerebral depletion of γ-aminobutyric acid.

REFERENCES

Dasgupta, S. R., Killam, E. K. & Killam, K. F. (1958). *J. Pharmacol.* **122**, 16*a*.
Killam, K. F. (1957). *J. Pharmacol.* **119**, 263.
Killam, K. F. & Bain, J. A. (1957). *J. Pharmacol.* **119**, 255.
Lindsay, D. M., Robson, J. M. & Sinclair, L. (1959). *Proc. Pharmacol. Soc.* (January).

Dorsal root reflexes in muscle afferent fibres. By J. C. Eccles, W. Kozak and F. Magni. *Department of Physiology, The Australian National University, Canberra, A.C.T., Australia*

As reported by Brooks, Koizumi & Malcolm (1955) and Brooks & Koizumi (1956) for the cooled animal, volleys in muscle afferents evoke reflex discharges into muscle afferents, which in general resemble the dorsal root reflexes (DRRs) that occur between cutaneous afferents (Barron & Matthews, 1935; Toennies, 1938). Both Group Ia and Ib afferent impulses are effective producers of DRRs, II and III being impotent, but the DRR occurs almost entirely in Ia fibres. Flexor afferents are much more effective than extensors, but both are equally good recipients. Ia and Ib volleys in the same or different nerves sum very effectively, simultaneous volleys in several flexor nerves producing small DRRs even at 38° C, and at 34° C large repetitive discharges virtually maximal for Ia. Central latency is about 5 msec and total duration up to 20 msec. With volley intervals longer than 10 msec summation gives place to depression, there being depressed DRRs with frequencies above 0·2/sec and complete suppression at 5–10/sec.

Group I muscle volleys produce a large prolonged potential field, the dorsal side of the cord being positive to the ventral, with a latency of about 4 msec and total duration of 100 msec. The relative effectiveness of muscle

volleys resembles their potency in producing DRRs. The topography of the potential field shows it to be produced by neurones of the intermediate nucleus, presumably by the after-hyperpolarization following spike discharge (Eccles, Eccles & Lundberg, 1960), the axonal terminals in the motoneuronal nucleus being sinks to the soma sources. Direct testing of excitability of muscle primary afferents by brief pulses through a microelectrode shows that a pronounced over-excitability of Ia fibres in the ventral horn follows the time course of the negative field there. Thus it would appear that the DRR is due to the depolarization which the potential field produces in the Ia afferent fibres in the ventral horn. Accommodation would account for the brief duration of the DRR and for depression with repetitive stimulation. However, it does not seem possible to reconcile several findings with this simple explanation of the DRR, a particular difficulty being that the over-excitability of the muscle afferent fibres extends right through the dorsal regions of the cord, where they would be anodally polarized by the potential field.

REFERENCES

BARRON, D. H. & MATTHEWS, B. H. C. (1935). *J. Physiol.* **85**, 73.
BROOKS, C. McC. & KOIZUMI, K. (1956). *J. Neurophysiol.* **19**, 61.
BROOKS, C. McC., KOIZUMI, K. & MALCOLM, J. L. (1955). *J. Neurophysiol.* **18**, 205.
ECCLES, J. C., ECCLES, R. M. & LUNDBERG, A. (1960). *J. Physiol.* (in the Press).
TOENNIES, J. F. (1938). *J. Neurophysiol.* **1**, 378.

The distribution of recurrent inhibition among motoneurones.

By ROSAMOND M. ECCLES, A. IGGO* and M. ITO. *Department of Physiology, The Australian National University, Canberra, Australia*

Samples of from seven to seventy-five of the motoneurones in the ventral horn nuclei supplying the hind limb were investigated by intracellular recording. The strength of the recurrent inhibition of each motoneurone was tested by firing single maximal antidromic volleys along individual muscle nerves in sequence and recording subsequent events in the motoneurone. The ten cats were lightly anaesthetized with Na pentobarbitol, the dorsal roots were cut and the spinal cord was divided at L2. The relation between the duration of the positive after-potential (AHP) and the conduction velocity of the motor axon, established by Eccles, Eccles & Lundberg (1958), was confirmed; in particular the motoneurones (tonic α motoneurones of Granit) supplying the soleus muscle had long after-hyperpolarizations (about 150 msec) and slow axonal conduction velocities (< 70 m/sec). When the aggregate inhibitory post-synaptic potential (IPSP) for individual cells, which was obtained by adding

* On leave from the Physiology Department, University of Edinburgh.

d

together the peak amplitudes of the IPSP generated by each muscle nerve alone, was plotted against the duration of the AHP, an almost linear relation was found, i.e. the longer the AHP the greater was the inhibitory potential. This means that the tonic α motoneurones are strongly inhibited by recurrent collaterals, whereas some of the phasic motoneurones are almost unaffected.

The relative sizes of the IPSP generated by different muscle nerves also depended on the position of a motoneurone in the spinal cord both longitudinally and dorsoventrally. The posterior biceps (PB) nucleus, which extends over about two segments, was systematically investigated. In one cat the IPSP generated in PB motoneurones was largest rostrally for anterior biceps volleys and largest caudally for medial gastrocnemius volleys. At any one level of the spinal cord there was, however, a highly consistent pattern, so that inadequate sampling could lead to an apparently meaningful result.

When the input pattern was calculated for the average cell of each nucleus and not for the individual cell, the effect of the position of the nucleus in the cord, although somewhat blurred, was still dominant. Two additional features were shown up by this analysis. First, individual nuclei were inhibited most strongly by their own axon-collaterals, with the striking exception of soleus, which was more strongly inhibited by adjacent nuclei than by itself. Secondly, some muscle nerves were very weak in causing inhibition, particularly plantar nerves, peroneal, flexor digitorum longus and soleus, whereas others, e.g. anterior biceps, medial and lateral gastrocnemius, were very effective.

These results suggest that, in addition to any direct influence of the muscle of origin, the recurrent collaterals have an important function in suppressing the tonic postural motoneurones. Such an action would be effective in removing the resistance of any postural tone in the red muscles of a limb when a quick movement was initiated by the phasic motoneurones.

Recurrent facilitation, in the form of a later small depolarization, was recorded very infrequently.

REFERENCE

Eccles, J. C., Eccles, R. M. & Lundberg, A. (1958). *J. Physiol.* **142**, 275–291.

Separation of slowly-exchanging fractions of the potassium of rabbit auricles. By A. Wilma Waddell and M. Weatherall. *Department of Pharmacology, London Hospital Medical College, London, E.* 1

Part of the potassium in rabbit auricles exchanges more slowly than the rest (Weatherall, 1959). The accelerated loss of ^{42}K known to occur from

auricles treated with 2:4-dinitrophenol (DNP) could theoretically result from failure to concentrate potassium in such a slowly-exchanging fraction (Weatherall, 1960). The enzymes sensitive to DNP are largely located in mitochondria (Brody, 1955) and it is possible that the slowly-exchanging potassium is in the same fraction of the tissue.

To test this hypothesis unseparated pairs of rabbit auricles were immersed in modified Krebs's saline at 37° C for 15 or 45 min. Sometimes the medium contained ^{42}KCl, or the auricles were transferred from inactive to ^{42}K medium; the period in tracer was always 15 min. In some experiments both inactive and active medium contained DNP (10^{-4} M). After immersion the auricles were rinsed briefly with inactive medium, cooled, chopped finely, ground with sand and suspended in sucrose (0·28 M)–sodium versene (0·01 M) adjusted to pH 7·4. Deposits were obtained from this initial suspension at 1000, 45,000, and 800,000 *g*-min, corresponding roughly to coarse tissue debris, sarcosomes and microsomes respectively. The coarse debris was resuspended and a second set of deposits obtained from it. All these operations were done at 0–2° C. Media were separated from deposit by decanting and finally with a fine pipette. The deposits were extracted with boiling water for 20 min, or ashed and dissolved, and the K, Na, and when appropriate the ^{42}K, of each fraction estimated. The Na content of the deposits was assumed to be due entirely to unremoved medium, and the ^{42}K and K contents were corrected accordingly.

The mean total K recovered from untreated pairs of auricles and the specific activity estimated by combining the K and ^{42}K for the various fractions agreed with previous estimations on unfractionated auricles, and showed that the recoveries of K and ^{42}K are satisfactory. The coarse deposit contained 10–13 % of the tissue K, virtually unexchanged. The high-speed deposits in all experiments contained measurable amounts of potassium, about 4 % of the total K of the tissue. The potassium in the deposits had a specific activity generally about one tenth of that in the residual supernatant. This specific activity was lower when the auricles had been immersed in inactive medium for 30 min than when they were put directly into ^{42}K medium. In the presence of DNP there was slightly less potassium in the deposits and the specific activity was significantly lower than in any controls.

These experiments show that part of the tissue potassium is associated with intracellular particles, that this potassium exchanges more slowly than the rest, and that uptake into the slow fraction or fractions is blocked by DNP.

REFERENCES

BRODY, T. M. (1955). *Pharmacol. Rev.* **7**, 335–363.
WEATHERALL, M. (1959). *J. Physiol.* **148**, 14*P*.
WEATHERALL, M. (1960). *J. Physiol.* **150**, 6*P*.

The spectral sensitivities of the clawed toad, *Xenopus laevis*, on white and black backgrounds. By PRISCILLA H. STRANGE. *Visual Research Division, Ophthalmological Research Unit, Institute of Ophthalmology, Judd Street, London, W.C.* 1

The spectral sensitivities of *Xenopus* on (*a*) a white background, and (*b*) a black background, have been compared by using partial contraction or expansion of the skin melanophores on the toes as a threshold criterion. On the white background the spectral sensitivity function is a simple broad curve, with a flat maximum at about 500 mμ; on a black background the spectral sensitivity is nearly the same as this, except in the region around 490 mμ, where there is a narrow range of wave-lengths for which the black background threshold is about 0·9 log. units less sensitive than the white. The results on a black background are in general agreement with Denton & Pirenne's results under these conditions (1954).

Dartnall (1956) separated two photosensitive pigments, maximal at 523 and 502 mμ, from the retina of *Xenopus*, present in the proportions 92 to 8%. The white-background sensitivity has a maximum compatible with the latter pigment, and the black-background sensitivity has the two-humped form interpreted by Dartnall as the modulus of the difference between the absorption spectra of the two pigments.

REFERENCES

DARTNALL, H. J. A. (1956). *J. Physiol.* **134**, 327–338.
DENTON, E. J. & PIRENNE, M. H. (1954). *J. Physiol.* **125**, 181–207.

The effect of neuraxial ablations upon the secretion of electrolyte-active steroid. By J. BLAIR-WEST, J. COGHLAN, D. A. DENTON, J. R. GODING, J. A. MUNRO and R. D. WRIGHT. *Department of Physiology, University of Melbourne, Australia*

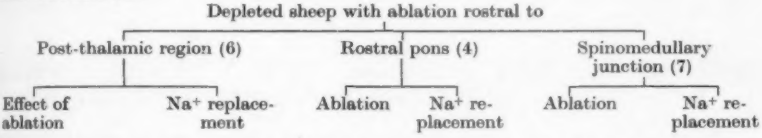
Emotional disturbance affects electrolyte-active steroid secretion (Denton & McDonald, 1957). Neural ablations affect aldosterone secretion during haemorrhage (Farrell, 1958). Depleted sheep and rats show highly selective appetites for Na salts (Richter 1958; Denton, Goding & Wright 1959, 1960). Thus the nervous system probably participates in the control of aldosterone secretion. Cross-circulation experiments in sheep and dogs (Denton, Goding & Wright, 1959) (Yankopoulos, Davis, Kliman & Peterson, 1959) show that aldosterone secretion is activated via a blood-borne stimulus in Na^+ depletion and caval constriction. Increased secretion could result from increase of an activator or decrease of an inhibitor.

The nervous system might affect the adrenal: (1) by secreting activating or inhibitory substances into the blood stream upon depletion or repletion

respectively; (2) by causing secretion of such substances from some other part of the body; (3) by modifying such secretion by some other part of the body: or (4) it might have no direct action at all.

With these propositions in view ablation experiments were carried out. The Na^+:K^+ ratio of the saliva was taken as the quantitative index of secretion of aldosterone (Denton, Goding & Wright, 1960; Coghlan, Denton, Goding & Wright, 1960).

Sheep were taken in two groups, Na^+ depleted and Na^+ replete. The plan of detection of removal of an activator or inhibitor in depleted sheep was as follows:



Most animals also underwent hypophysectomy. A similar plan was followed for Na^+ replete sheep, with observation of the effect of Na^+ depletion induced after the ablation.

The results were:

(1) With two qualified exceptions in spinomedullary ablation, no ablation in depleted sheep caused the salivary Na^+: K^+ ratio to rise to normal or post-adrenalectomy levels. The brain is therefore not the *sole* source of activation of aldosterone secretion.

(2) No ablations caused the salivary Na^+: K^+ ratio of replete sheep to fall, within 6 hr. The brain is therefore not a sole source of an inhibitor, the removal of which increases aldosterone secretion.

(3) The only preparations in which Na^+ repletion after ablation caused a rapid increase in the Na^+: K^+ ratio were two with ablation rostral to the posterior thalamus.

(4) There were episodes in which salivary Na^+: K^+ fell subsequent to each of these types of ablation, indicating that neither the encephalon nor the pituitary is essential for this to occur. With the more anterior ablations fall in the Na^+: K^+ ratio occurred in association with developing Na^+ deficiency, but with spinomedullary ablation decreases occurred without Na^+ deficiency.

(5) Changes in salivary Na^+: K^+ ratio occurred after ablations of all types, but were not consistent with any simple explanation. The variability may have been due to irritative, paralytic, or release phenomena in the residual nervous tissue, or to other mechanisms of activation, either persisting or induced by the experimental procedures.

REFERENCES

COGHLAN, J. P., DENTON, D. A., GODING, J. R. & WRIGHT, R. D. (1960). *Post Grad. Med. J.* **36**, 76–103.

DENTON, D. A., GODING, J. R. & WRIGHT, R. D. (1959). *Brit. med. J.* ii, pp. 447–456 and 522–530.

DENTON, D. A., GODING, J. R. & WRIGHT, R. D. (1960). *Clinical Endocrinology*, **1**, ed. E. B. ASTWOOD, pp. 373–391. New York: Grune and Stratton.

DENTON, D. A. & MCDONALD, I. R. (1957). *J. Physiol.* **138**, 44–62.

FARRELL, G. (1958). *Physiol. Rev.* **38**, 709–728.

RICHTER, C. P. (1956). *L'Instinct dans le comportement des animaux et de l'homme*, pp. 577–632. Paris: Masson et cie.

YANKOPOULOS, N. A., DAVIS, J.-O., KLIMAN, B. & PETERSON, R. E. (1959). *J. clin. Invest.* **38**, 1278–1289.

Epidermis as a thick membrane. By R. T. TREGEAR. *Chemical Defence Experimental Establishment, Porton Down, Salisbury, Wilts*

Treherne (1956) found that the epidermis of excised rabbit skin behaved as a lipoidal membrane of definite storage capacity when organic substances were allowed to diffuse through it. In the present experiments an organic liquid, tri-*n*-butyl phosphate (TBP), labelled with ^{32}P, was placed in 1 mg drops either on intact skin or on excised skin superfused on the dermal surface with Ringer's solution; the penetration of the TBP was estimated from its radioactivity. In both preparations the penetration was not immediate, but the rate rose exponentially to a steady level, indicating the presence of some storage capacity. The time constant of the rise (τ) and the steady level reached (p) varied widely between the species tested and the modes of preparation used, but the multiple $p\tau$ was comparatively constant (Table 1). Since $p\tau$ should, on a simple theory, be proportional to the storage capacity per unit area of skin, this constancy suggests that both pig and rabbit skin have similar storage capacities despite the difference in their epidermal structure.

The effect of circulatory changes on skin penetration was examined in perfused rabbit thigh skin (Feldberg & Paton, 1951). Above a perfusion flow of approx. 10 μl./min/cm^2 of perfused skin the penetration rate was

TABLE 1. Penetration of TBP through excised and intact flank skin: time constant of the exponential rise (τ) and the steady penetration reached (p)

Species		τ (min)	p (μg/cm^2/min)	$p\tau$
Rabbit	Intact	15*	2·20	33
	Excised	80	0·43	34
Pig	Intact	80	0·34	27
	Excised	300	0·12	36

Each result is the mean of 4–8 experiments. * Measured on perfused skin.

only slightly dependent on the flow, indicating that the TBP concentration beneath the epidermis was negligible. As this flow was achieved with only

moderate perfusion pressures (25–50 mm Hg) it may normally occur in warm skin; this would explain why intact skin was more penetrable than excised skin (Table 1).

REFERENCES

FELDBERG, W. & PATON, W. P. (1951). *J. Physiol.* **114**, 490–509.
TREHERNE, J. E. (1956). *J. Physiol.* **133**, 171–180.

The effect of anoxia produced by removal of red cells on aldosterone secretion from the dog adrenal. By MARGARETHE HOLZBAUER* and MARTHE VOGT. *A.R.C. Institute of Animal Physiology, Babraham, Cambridge*

The haematocrit was lowered in dogs (anaesthetized with chloralose) by simultaneous exchange of blood for homologous plasma, thus causing anaemia while leaving the circulatory blood volume unchanged. By exchanging 40 ml./kg the cell volume was reduced from about 50 to approximately 25%. This led immediately to an increase in secretion of aldosterone estimated in samples of adrenal vein blood. The effect was abolished by cutting the splanchnic nerves. It must be due to stimuli acting on structures other than volume receptors.

Evidence for the presence of an inhibitory transmitter substance in Factor I extracts. By H. MCLENNAN. *Department of Physiology, University of British Columbia, Vancouver, Canada*

The properties of extracts of mammalian central nervous system which are able to inhibit certain synaptic processes have been the subject of various publications in recent years. The suggestion that these extracts, said to contain Factor I, might owe some at least of their actions in mammals to the presence of a transmitter of inhibitory neurones was made in part as a result of the demonstration by Florey & McLennan (1955) that Factor I reversibly inhibited tendon-jerk reflexes, and that the effect was prevented by small doses of strychnine.

Curtis, Phillis & Watkins (1959) have given criteria for the electrical effects to be expected if a solution containing an inhibitory transmitter should be applied to a single neurone. An increase in the resting membrane potential, with a concomitant decrease or abolition of evoked inhibitory post-synaptic potentials (IPSP) and diminished excitatory post-synaptic potentials, should be observed.

In the present study Factor I solutions have been applied to the exposed spinal cord of cats, and records obtained with glass microelectrodes from extensor motoneurones. Post-synaptic potentials were

* Beit Memorial Research Fellow.

evoked by stimulation of appropriate dorsal-root filaments. In conformity with the criteria of Curtis *et al.*, Factor I increased the resting membrane potential by 2–6 mV and invariably reduced the amplitude of an evoked IPSP. EPSP's were little affected if the alteration in membrane potential was small, and reduced when larger membrane potential changes were observed. It is concluded that these observations provide strong evidence for the presence of an inhibitory transmitter in the Factor I extracts.

This work was supported by a grant from the National Research Council of Canada.

REFERENCES

Curtis, D. R., Phillis, J. W. & Watkins, J. C. (1959). *J. Physiol.* **146**, 185–203.
Florey, E. & McLennan, H. (1955). *J. Physiol.* **130**, 446–455.

The different effects of aldosterone and cortisol in recumbency.
By J. N. Mills, S. Thomas and K. S. Williamson. *Department of Physiology, University of Manchester*

It has previously been shown (Mills, Thomas & Williamson, 1960) that aldosterone increases potassium and hydrion excretion, whereas hydrocortisone, in doses followed by a similar sodium retention, causes a much bigger rise in potassium and often a fall in hydrion excretion. These differences can be further explored if the steroids are injected when the subject awakes in the morning, and he remains recumbent until midday. In these circumstances, in control experiments, sodium, chloride, and potassium excretion was high and hydrion excretion low. Two such control experiments, and two with injection of each steroid, 0·5 mg aldosterone and 100 mg cortisol, were performed upon each of two subjects.

Aldosterone injection was followed by the usual low sodium excretion, but potassium excretion was unaltered, and hydrion excretion raised more than in any other circumstance we have investigated; chloride excretion sometimes fell.

Cortisol injection was followed by a less consistent lowering of sodium, but a much bigger rise in potassium, excretion; hydrion excretion was unaltered or reduced, and in one subject urinary pH rose to 7·50 and 7·60.

These observations support our earlier tentative suggestion, that aldosterone promotes sodium reabsorption either in association with chloride or by cation exchange for potassium or hydrion, whereas cortisol promotes only the sodium $\rightleftharpoons$ potassium exchange mechanism.

REFERENCE

Mills, J. N., Thomas, S. & Williamson, K. S. (1960). *J. Physiol.* **151**, 312–331.

Muscle spindle receptors. By K. Diete-Spiff. *Department of Physiology, University College London*

Hunt (1954) stimulated tetanically the motor nerve fibres to the intrafusal muscles in isolation from the fibres to the extrafusal muscles. He showed that both types of spindle receptors, primary and secondary, were excited, and concluded that the difference in behaviour between the receptors was merely quantitative; the primary endings were slightly more sensitive to stretch than the secondary endings. In the present work the effects of selective activation of the intrafusal muscle fibres have been re-investigated.

The experiments were made on rabbits, decerebrated or anaesthetized either with urethane or sodium hexobarbitone. Stimuli were applied to either the nerve to the plantaris muscle or to the nerve to the medial head of the gastrocnemius muscle: all other nerves in the leg were cut. The muscle contractions and single spindle receptor discharges from dorsal root filaments were recorded electrically.

With single maximal shocks to the muscle nerve both types of receptor paused during the rising phase of the muscle contraction. Supramaximal shocks caused the fibres of high conduction velocity to discharge during this phase and they have been classified as coming from the primary endings. The slower fibres could not be made to discharge during the rising phase of the contraction by any strength of stimulus, and these fibres have been assumed to come from the secondary receptors (Matthews, 1933).

Succinylcholine, 25–100 μg by retrograde injection via the posterior tibial artery, blocked the muscle contraction, but single supramaximal motor-nerve shocks were able to evoke discharges from the primary endings. It was still not possible to activate the secondary endings by this means; for these to be excited tetanic stimulation was necessary. It is concluded that even in the absence of extrafusal shortening, single motor-nerve shocks produce insufficient intrafusal shortening to excite the secondary receptors. This might be ascribed to the lower sensitivity of these endings to stretch. However, the same result is obtained no matter what the initial tension of the muscle. It is suggested, in agreement with Boyd (1959), that the secondary endings lie on slow muscle capable of graded responses, and that several stimuli are necessary to produce shortening adequate to excite the receptors.

REFERENCES

Boyd, I. A. (1959). *J. Physiol.* **145**, 55–56*P*.
Hunt, C. C. (1954). *J. gen. Physiol.* **38**, 117–131.
Matthews, B. H. C. (1933). *J. Physiol.* **78**, 1–53.

The applicability of Hodgkin & Huxley's (1952) formulation of the ionic hypothesis to cardiac muscle. By D. NOBLE. *Department of Physiology, University College London*

Interrelationships between mitochondria and the endoplasmic reticulum bearing on a theory of the origin of mitochondria. By J. D. ROBERTSON. *Department of Anatomy, University College London*

Mitochondria consist essentially of a matrix material (*m.m.*, Fig. 7) bounded by two unit membranes (arrows, Fig. 7) (Robertson, 1959) separated by a gap $\sim$ or $<$ 75 Å wide. The inner membrane is enfolded to make the cristae (Palade, 1953).

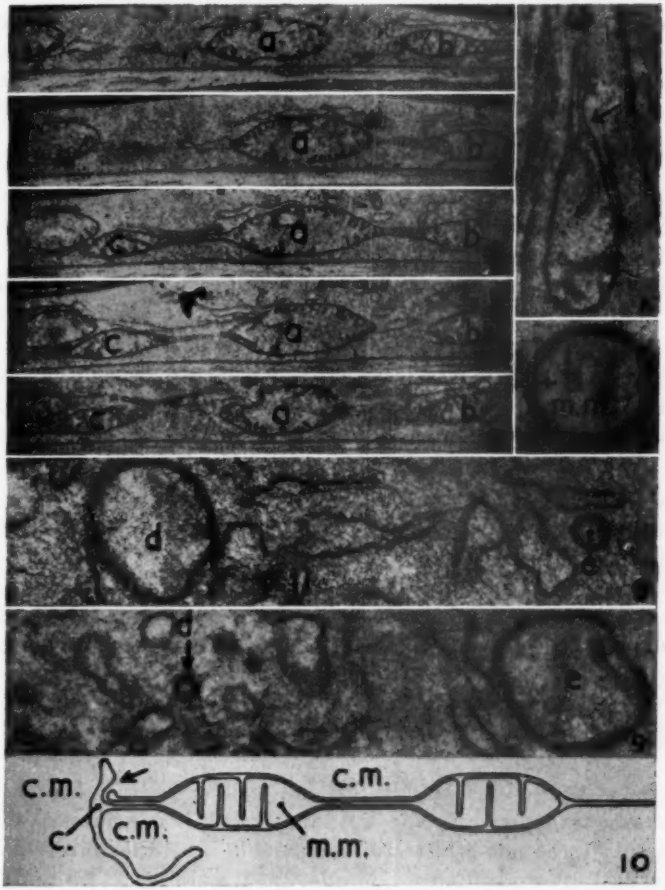
In Schwann cells of myelinating mouse sciatic nerves mitochondria are arranged in chains spaced $\sim 2\,\mu$ apart and interconnected by tubular double-walled necks $\sim$ 600–800 Å in diameter. Figures 1–5 are serial longitudinal sections through a chain of three mitochondria (*a–c*) establishing this point. Figure 8 is a transection through such a chain including a mitochondrion *d* and the neck of another mitochondrion *e*. Figure 9 is a successive section to Fig. 8 somewhat deeper in the same Schwann cell, including the same area but now passing through the neck of mitochondrion *d* and the body of mitochondrion *e*. Both membranes are visible in the necks (Fig. 8 *e*). The necks thus maintain continuity between the matrices and the inner and outer membranes of each mitochondrion in a chain. Continuity between outer mitochondrial membranes and tubular or membranous components of the endoplasmic reticulum (ER) have been found in developing axons (arrow, Fig. 6) and in adult pancreatic acinar cells.

A hypothesis of the origin of mitochondria based on these findings is presented diagrammatically in Fig. 10. According to this, mitochondria develop by extension of a unit membrane-bounded finger-like process of the cytoplasmic matrix (*c.m.*) into a cisterna of the ER (arrow, Fig. 10). This extends and expands to make the mitochondrial matrix (*m.m.*) and its bounding unit membrane folds to make the cristae. The gap between the membranes is thus continuous at times with the cisternal contents (*c*). of the ER and may be a site of formation of secretory material. This is supported by certain appearances of mitochondria in pancreatic acinar cells.

To what extent the membrane continuities demonstrated are maintained in time is unknown. The connexions may pinch off and be reformed. Nevertheless, it appears that the mitochondrial matrix is simply a small segregated pocket of the cytoplasmic matrix.

REFERENCES

PALADE, G. E. (1953). *J. Histochem. Cytochem.* **2**, 188.
ROBERTSON, J. D. (1959). *Biochem. Soc. Symp.* **16**, 3–43.



Magnifications: Figs. 1–5, × 17,000; Fig. 6, × 50,000; Fig. 7, × 105,000, Figs. 8–9, × 86,000.

Movement of a dye in the spinal subarachnoid space. By H. F. GRUNDY. *Department of Pharmacology, University of Cambridge*

By means of an iron framework and clamps, anaesthetized cats were held fixed in a prone position with the vertebral canal horizontal. A marker dye was introduced under standard conditions into the subarachnoid space at

the first sacral, eleventh thoracic or seventh cervical vertebral level. The animals were killed by intravenous chloroform within 10 min, at 85 min or 4 hr after the dye introduction. In half of the 85 min series of experiments convulsions were induced during the last 30 min by the administration of picrotoxin. After death the head and vertebral column of the cat were isolated and rapidly frozen in a solid CO_2–ethanol mixture, the horizontal position being maintained during these manoeuvres. The frozen specimen was sectioned transversely by means of a band-saw and the limits of dye spread determined by direct observation.

It was found that even after 4 hr the dye had moved only slightly from its site of introduction but that much greater movement was induced by picrotoxin convulsions. It is concluded that in general there is little cranial $\rightleftarrows$ caudal circulation of cerebrospinal fluid in the spinal subarachnoid space of the horizontal anaesthetized cat. However, movement of the fluid in this space is markedly increased by picrotoxin convulsions.

The energy requirements of treadmill walking in relation to sex and athletic training. By J. E. Cotes, J. Drummond, F. Meade and C. Yandle. *Medical Research Council Pneumoconiosis Research Unit, Llandough Hospital, near Penarth, Glamorganshire*

Working at Cambridge, Booyens & Keatinge (1957) found a low energy requirement for women during track walking, which they attributed to the women performing less work in lifting their bodies than men. This is consistent with the women having shorter leg and pace lengths (Cotes, Meade & Wise, 1958). But Durnin (1958) found equal energy expenditures for men and women, and Ralston (1958) noted that values reported for men by Booyens were rather high; other published literature confirms this. However, we observed comparable values in untrained male recruits during treadmill walking and these fell with training (Cotes & Meade, 1959).

We have studied eight middle-distance male and seven short-distance female athletes, participants in the 1958 British Commonwealth Games, and twenty-three non-athletes of comparable age. Energy expenditure and vertical lift work (Cotes & Meade, 1960) were measured during the 4th to 6th minute of walking for the first time on a treadmill at 2–4 m.p.h. (3·2–6·4 km/hr).

Energy expenditures of the men, both athletes and non-athletes, were very similar to those of the Cambridge men and male Army recruits before training (Fig. 1), but values for the women lay along the regression line obtained for the recruits *after* training. (Energy expenditure = $4{\cdot}25 \times 10^{-6} V^2(\mathrm{m/min})^2 + 0{\cdot}0386$ kcal/min/kg body weight; where V = velocity (m/min).) This line relates energy expenditure and velocity for subjects

walking at 1 to 3½ or 4 m.p.h., the upper limit being influenced by the subject's leg length and hip-joint mobility.

The findings suggest that the energy expended in treadmill walking by untrained males may be excessive at first, irrespective of their level of physical fitness, and falls with practice. Females tend to walk more economically from the start.

Copies of experimental protocols may be obtained from the authors.

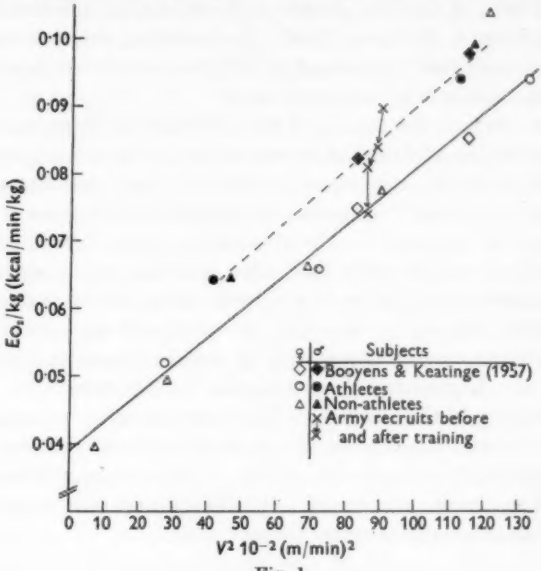


Fig. 1

REFERENCES

Booyens, J. & Keatinge, W. R. (1957). *J. Physiol.* **138**, 165–171.
Cotes, J. E. & Meade, F. (1959). *Ergonomics*, **2**, 195–206.
Cotes, J. E. & Meade, F. (1960). *Ergonomics*, **3**, 97–120.
Cotes, J. E., Meade, F. & Wise, M. E. (1958). *J. Physiol.* **141**, 28–29*P*.
Durnin, J. V. G. A. & Namyslowski, L. (1958). *J. Physiol.* **143**, 573–578.
Ralston, H. J. (1958). *Fed. Proc.* **17**, 127.

Histamine and gastric secretion in man. By C. G. Clark, V. June Curnow, J. G. Murray, F. O. Stephens and J. H. Wyllie*.
Departments of Surgery, Aberdeen University, and King's College Hospital, London, S.E. 5

In order to carry out valid quantitative tests of gastric secretion, two conditions are desirable: (1) application of a maximal stimulus to the

* Supported by a grant from the Medical Research Council.

secretory cells of the stomach; and (2) collection of all gastric secretions over timed periods. Kay (1953) prevented systemic effects of histamine by giving mepyramine maleate intramuscularly, and showed that subcutaneous injection of 0·04 mg/kg body weight of histamine acid phosphate caused acid secretion at a rate which was not exceeded by giving still larger doses of histamine. Providing the tip of the collecting tube is accurately located in the antrum of the stomach and appropriate continuous suction applied, 90–100 % of the total gastric contents can be collected over timed periods (Johnston & McCraw, 1958). Under these circumstances, it has been demonstrated that the output of HCl in consecutive tests is, within narrow limits, constant for any individual.

We have confirmed the results of this Augmented Histamine Test in a series of patients. In addition, impressively large amounts of pepsin appear in the gastric juice. We compared, in the same patients, the amounts of HCl and pepsin secreted in response to Augmented Histamine Tests with those secreted in response to effective insulin tests. Acid output after histamine agreed closely with that after insulin; pepsin secretion after histamine was consistently about two-thirds of that after insulin. Hexamethonium iodide (25 mg) or atropine sulphate (0·6 mg) given by intramuscular injection caused about 50 % reduction of acid and pepsin secretion in both the Augmented Histamine and insulin tests.

It is generally held that histamine has a specific effect in causing gastric secretion by acting directly on the parietal cells of the stomach. The present results indicate that the action of histamine in these doses in man is neither specific nor direct. It is suggested that histamine acts, at least in part, through nervous tissue.

REFERENCES

KAY, A. W. (1953). *Brit. med. J.* ii, 77–80.
JOHNSTON, D. H. & MCGRAW, B. H. (1958). *Gastroenterology*, **35**, 512–516.

The action of a 'hemicholinium' compound (HC3) on the central inspiratory discharge and diaphragm activity in the cat. By R. HOLMES and H. WILSON. *Chemical Defence Experimental Establishment, Porton Down, Salisbury, Wilts, and Department of Pharmacology and General Therapeutics, University of Liverpool*

The lethal action of a hemicholinium compound (HC3),α,α′-dimethylaminoethanol-4,4′biacetophenone (Long & Schueler, 1954; Schueler, 1955) was ascribed by Schueler (1955) to a slowly developing paralysis of the central respiratory mechanisms. Kasé & Borison (1958) concluded that this action was exerted on the brain-stem mechanisms and claimed that the neuromuscular block produced by large doses only enhanced the central

effect. Longo (1959), however, showed that HC3 had no central action and that the effects were due to neuromuscular block of the respiratory muscles. A neuromuscular blocking action of HC3 was also reported by Reitzel & Long (1959) and Wilson & Long (1959).

In the present study the action of HC3 on respiration has been re-examined using cats anaesthetized with pentobarbitone, vagotomized and artificially ventilated so that spontaneous respiratory movements continued. Medullary inspiratory activity was recorded from the region of the obex by inserting a micro-electrode made from 5 or 10 μ platinum wire insulated except at the tip by a thin glass sheath. Bipolar needle electrodes were used for recording diaphragm muscle action potentials. HC3 was given in doses ranging from 2 to 4 mg/kg I.V.

Respiratory activity was affected little by the smaller dose of HC3. Larger doses (3 or 4 mg/kg), produced initially a temporary increase in the discharge of inspiratory and diaphragm units. This was probably due to a rapid fall in blood pressure which followed the injection. The pressure returned to the pre-injection level within a few minutes and spontaneous respiratory activity also became normal at this time. The subsequent effect of HC3 was a slowly developing neuromuscular block at the diaphragm which became marked at about 20–30 min and which was not associated with inhibition of central inspiratory activity. The reduction in diaphragm action potentials was not due to a decrease in the intensity of the inspiratory discharge, for when this was increased by reducing the artificial ventilation the return of activity in some diaphragm units was quickly followed by sporadic firing and complete neuromuscular block: the discharge of the 'inspiratory centre' continued at a high intensity.

In no case in sixteen experiments with HC3 has an inhibition of central respiratory activity been observed. Thus 131 min after 4 mg/kg HC3 I.V. the discharge of inspiratory neurones was unimpaired, although block at the diaphragm was practically complete. In another experiment, the central discharge was still unaffected 50 min after a third dose of 2 mg/kg, the first dose having been given 3 hr 51 min earlier.

These results indicate that HC3 has no effect on the activity of the respiratory centre and that it produces a neuromuscular block at the diaphragm. It appears that death in animals receiving lethal doses of the drug is probably due to asphyxia resulting from paralysis of the respiratory muscles.

REFERENCES

KASÉ, Y. & BORISON, H. L. (1958). *J. Pharmacol.* **122**, 215.
LONG, J. P. & SCHUELER, F. W. (1954). *J. Amer. Pharm. Ass.* (Sci. ed.), **43**, 79.
LONGO, V. G. (1959). *Arch. int. pharmacodyn.* **119**, 1.
REITZEL, N. L. & LONG, J. P. (1959). *Arch. int. pharmacodyn.* **119**, 20.
SCHUELER, F. W. (1955). *J. Pharmacol.* **115**, 127.
WILSON, H. & LONG, J. P. (1959). *Arch. int. pharmacodyn.* **120**, 343.

The action of some sympathomimetic amines on the cat's iris and blood vessels. By E. MARLEY*. *Department of Pharmacology, Royal College of Surgeons, Examination Hall, Queen Square, London, W.C.* 1

The effects of some sympathomimetic amines were compared on the normal and on the chronically denervated iris of the chloralosed cat (superior cervical ganglion and post-ganglionic trunk removed 10–14 days previously) and a threefold classification of activity, reminiscent of that proposed by Fleckenstein & Burn (1953) for the effect of sympathomimetic substances on the nictitating membrane, was possible. Thus (1) the normal mydriatic action of the phenylethylamines was absent on the denervated iris; (2) for substances with an —OH either on the benzene ring, or on the β-carbon atom of the side-chain, the threshold dose required to dilate the pupil of the denervated iris was raised; and (3) for substances with two —OH groups on the benzene ring, or one —OH on the benzene ring together with an —OH on the β-carbon, the denervated iris was supersensitive for threshold dose and for degree and duration of mydriasis. All three groups of drugs dilated the pupil after degeneration of the parasympathetic post-ganglionic innervation to the iris, or after section of the midbrain interrupting the sympathetic connexions from the hypothalamus to the iris.

Recently, Burn & Rand (1958, 1959) reported that after chronic reserpine administration, the cat's iris and blood vessels became supersensitive to adrenaline and noradrenaline but insensitive to tyramine, a condition they likened to that following chronic post-ganglionic sympathetic denervation.

In the present experiments, after chronic intraperitoneal reserpine treatment (0·5 mg/kg for 2 days, 1·0 mg/kg third day) the mydriatic action of groups (1) and (2) of the sympathomimetic drugs was reduced or absent. In contrast with the chronic denervation experiments and those of Burn & Rand (1959), the sensitivity of the iris to the catechols was not increased except in one of the seven chronic reserpine experiments, in which a massive intraperitoneal dose (3 mg/kg first day, 2 mg/kg second day) was given. Enhanced pressor responses to the catechols were invariably found, as were reduced blood-pressure responses to the other two groups of drugs.

Immediately after intravenous reserpine (0·5–1·0 mg/kg) the mydriatic action of all the sympathomimetics was diminished. As noted by Bein, Gross, Tripod & Meier (1953) the pressor response to the catechols gradually increased, but this also developed during repeated intravenous administration of the catechols without reserpine. In all the reserpine experiments,

* Holder of M.R.C. Clinical Research Fellowship.

after cocaine (0·2 mg/kg intravenously) the action of the catechol substances on the blood vessels and innervated pupils was augmented, the effect of other sympathomimetics, if present, being reduced.

Thus the analogy between the effect of reserpine and sympathetic denervation proved consistent only for the reduced response of the iris and blood vessels to the sympathomimetics other than those of group (3).

REFERENCES

Bein, H. J., Gross, F., Tripod, J. & Meier, R. (1953). *Schweiz. med. Wschr.* **83**, 1007–1012.
Burn, J. H. & Rand, M. J. (1958). *J. Physiol.* **144**, 314–336.
Burn, J. H. & Rand, M. J. (1959). *J. Physiol.* **147**, 135–143.
Fleckenstein, A. & Burn, J. H. (1953). *Brit. J. Pharmacol.* **8**, 68–78.

A pulsating cuff technique for determining an index of regurgitation in aortic incompetence. By I. F. S. Mackay and J. F. Palmer. *Department of Physiology, University College of the West Indies, Jamaica*

The purpose of this technique is to provide a measurement of the degree of regurgitation in aortic incompetence.

The basis of the technique is to use a pneumatic cuff placed on the upper arm in which pressure could be rapidly applied and released at predetermined times during the cardiac cycle. The cuff pressure (200–220 mm Hg) was applied during the diastolic phase and thus should stop any regurgitation during this period of the cardiac cycle. The pressure was released to zero during the period of ejection and thus should not have caused an obstruction to flow during this phase. The forearm-hand volume changes were measured plethysmographically while the subject was supine and the arm was held in an upward position at an angle of 20° from the vertical. The ambient temperature used was approximately 30° C. The pneumatic cuff was connected by a wide-bore tube ($\frac{1}{2}$ in., 12 mm) and a sliding valve to a pressure reservoir. The opening and closing of this sliding valve was timed from the R-wave of the electrocardiograph, and may be delayed or varied in duration. A second cuff placed close to the plethysmograph was inflated to a steady pressure of 50 mm Hg. The rate of increase in forearm-hand volume (blood flow) is recorded during the operation of this pulsating cuff. The index of regurgitation is calculated from the increase in flow obtained by the pulsating cuff as compared with that measured by venous occlusion plethysmography using a venous occluding pressure of 50 mm Hg.

This approach has been compared with an earlier method (Mackay, 1960), where steady cuff pressures were employed, and has been found to be more effective in increasing the blood flow.

e

The same measurements have also been made on normal subjects for comparison. Here, when the pulsating cuff is used, an increase in forearm blood flow may often be demonstrated, but this is much smaller than that which was measured in subjects with aortic incompetence.

The authors wish to thank Baum and Co. of New York for making the special pressure cuff.

REFERENCE

MACKAY, I. F. S. (1960). *Amer. J. Cardiol.* **5**, 321–327.

The influence of ionic strength on the anaphylactic reaction in guinea-pig lung. By K. F. AUSTEN* and W. E. BROCKLEHURST. *National Institute for Medical Research, Mill Hill, London, N.W. 7*

The antigen-induced release of histamine from perfused, chopped guinea-pig lung is extremely sensitive to acute changes in the ionic strength of the suspending medium. When the NaCl concentration of Tyrode solution was increased to 10·0 g/l. (0·195 M) from 8·0 g/l. (0·161 M), the histamine release fell by 30–48 %. When the NaCl concentration was reduced to 6·0 g/l. (0·125 M), the histamine release increased by 48–61 %. These results were obtained when antigen was added to sensitized tissue immediately after the suspending medium had been changed from normal Tyrode to NaCl-augmented or NaCl-depleted Tyrode solution. Incubation of the tissue in the modified solutions for 5 min before addition of antigen did not alter the respective inhibition or enhancement.

When the molarity of salt-depleted Tyrode solution (6·0 g/l.) was increased by adding $MgCl_2$, much greater inhibition was produced at each final molarity than when the same molarity was achieved by adding NaCl. On the other hand, alterations of molarity by the addition of glucose or sucrose produced very little inhibition. These studies indicate that it is cationic rather than anionic concentration which is critical. It was not possible to confirm this by the use of divalent anions, because such anions could inhibit by combining with calcium which is essential to the anaphylactic reaction.

The sensitivity of the anaphylactic release of histamine from guinea-pig lung to changes in the salt concentrations of Tyrode solution is remarkably similar to the effect of variation in salt concentration on the lysis of the sensitized red cell—carrying the first, fourth, and second components of complement (EAC′ 1, 4, 2)—by the third component (C′ 3) (Becker & Wirtz, 1959). This reaction between the EAC′ 1, 4, 2 cell and C′ 3 is also inhibited by salicylaldoxime (Mills & Levine, 1959). When salicylaldoxime (0·5 mM) was added to the lung tissue 10 sec before antigen there was a 50 % inhibition of the histamine release.

* U.S.P.H.S. Postdoctoral Research Fellow.

These studies suggest that the anaphylactic release of histamine from guinea-pig lung requires a factor similar to C′ 3. However, even if C′ 3 itself participates, it must be in addition to the heat-labile, calcium-requiring factor or factors known to be necessary for the anaphylactic release of histamine from guinea-pig lung (Mongar & Schild, 1957).

REFERENCES

BECKER, E. L. & WIRTZ, G. H. (1959). *Biochem. biophys. acta*, **35**, 291–292.
MILLS, S. E. & LEVINE, L. (1959). *Immunology*, **2**, 368–383.
MONGAR, J. L. & SCHILD, H. O. (1957). *J. Physiol.* **135**, 301–338.

Calorigenic activity of catechol derivatives in new-born cats. By R. E. MOORE and MARY C. UNDERWOOD. *Department of Physiology, Royal Free Hospital School of Medicine, London, W.C.* 1

We have previously reported the effect of noradrenaline (NAd) in raising the heat production of new-born kittens (Moore & Underwood, 1959, 1960). A considerable rise in oxygen consumption, rectal and skin temperatures followed the subcutaneous (S.C.) injection of 400 μg NAd/kg at an environmental temperature of 35° C in the first few weeks of life.

It is of some interest to know what type of compound shares with NAd this calorigenic activity, especially those with known cardiovascular actions and those concerned in NAd biosynthesis and inactivation. The activity of some of these compounds, administered S.C., 400 μg/kg, at 35° C is summarized below:

(*a*) *Noradrenaline series*, i.e. terminal —NH_2 on the side chain.
Noradrenaline (+ +)
Normetanephrine (0)
'Novadral' (Diwag A. G.) (0)

(*b*) *Adrenaline series*, i.e. terminal —$NH.CH_3$ on the side chain.
Adrenaline (±)
Metanephrine (0)
Phenylephrine (±)

(*c*) *Isoprenaline series*, i.e. terminal —$NH.CH(CH_3)_2$ on the side chain.
Isoprenaline (+ +)
'DCI' (+) (Lilly 20522; substitution of —Cl for —OH on the isoprenaline ring)

(Calorigenic response indicated by + +, +, ±, 0 ranging from high to no activity, with S.C. injection of 400 μg/kg at 35° C.)

It is interesting that the activities do not accord entirely with their known cardiovascular effects in the adult. Transient cardiovascular

activity before blocking has been observed with DCI (Moran & Perkins, 1958) but in our experiments DCI does not block the calorigenic effect of isoprenaline.

If dopamine is a biological precursor of NAd and normetanephrine the first metabolite of NAd (Axelrod, 1959), then the pattern of calorigenic activity would implicate NAd as the active substance, for whereas dopamine has some activity, *O*-methylation inactivates NAd.

The general picture presented suggests that cell penetration is one determining factor and that the new-born kitten may differ from the adult in this respect.

REFERENCES

AXELROD, J. (1959). *Pharmacol. Rev.* **11**, 402–408.
MOORE, R. E. & UNDERWOOD, M. C. (1959). *J. Physiol.* **150**, 13–14*P*.
MOORE, R. E. & UNDERWOOD, M. C. (1960). *J. Physiol.* (in the Press).
MORAN, N. C. & PERKINS, M. E. (1958). *J. Pharmacol.* **124**, 223–237.

Pulse wave velocity in pulmonary vessels. By C. G. CARO* and D. A. MCDONALD. *Department of Physiology, St Bartholomew's Hospital Medical College, London, E.C.* 1

A knowledge of wave velocity in the pulmonary arteries is essential to the understanding of the behaviour of pulsatile pressure and flow. Measurements were made in isolated rabbit lungs which were perfused with isotonic saline. Sinusoidal oscillatory flow was generated by a pump over a frequency range of 1·0–14·0 c/s. To exclude the imposed oscillatory flow from the perfusion reservoir it was maintained at a pressure of 2 atm. and linked to the system through a reducing valve which regulated steady flow. Mean arterial pressure was maintained at 10 mm Hg. A glass cannula was inserted in the pulmonary trunk; a capacitance manometer was attached to a side arm. A second manometer recorded the pressure in a distal artery through a nylon catheter. This was passed through the pulmonary trunk until it wedged in a peripheral vessel, then forced through the lung surface. It was withdrawn a measured distance and secured. Intervals used ranged from 3·3 to 5·5 cm. An harmonic resolver (Taylor, 1959) running at the pump frequency recorded the amplitudes of the sine and cosine components of the pressures at the two points. The phase angles of the proximal and distal pressure wave were thus given. The phase velocity was calculated from the phase difference.

Ten experiments were performed in six animals. The mean phase velocity over the range 6–10 c/s was $89{\cdot}5 \pm 8{\cdot}6$ (S.E.) cm/sec. Above 10 c/s the average was slightly higher (105 cm/sec), but the oscillatory pressure was then as great or greater than the mean. At low frequencies the

* On leave from the Department of Medicine, Queen Elizabeth Hospital, Birmingham.

apparent phase velocities recorded were very much higher, falling from values *ca.* 500 cm/sec at 1 c/s to a minimum, usually slightly less than the average, between 4–6 c/s. This behaviour is due to reflexion, as demonstrated previously in systemic arteries (McDonald & Taylor, 1959). This is consistent with the average wave velocity and indicates that the pulmonary arterial bed is about a quarter wave-length around 4 c/s. The reason that the phase velocities above this frequency are relatively stable is that they are being measured over larger fractions of a wave-length and any reflexion effects are averaged out.

The pulse wave velocity over the normal frequency range of the rabbit pressure pulse (pulse rate 3–6 c/s) is thus found to be rather less than 1 m/sec compared to the derived value of 2 m/sec assumed by Engelberg & DuBois (1959). We also see that, in terms of wave-length, the pulmonary and systemic systems are of comparable length.

Oscillatory pressures were also recorded in the pulmonary veins, thus agreeing with the observations of Lee & DuBois (1955) that pulmonary capillary flow in man is pulsatile.

REFERENCES

Engelberg, J. & DuBois, A. B. (1959). *Amer. J. Physiol.* **196**, 401–414.
Lee, G. de J. & DuBois, A. B. (1955). *J. clin. Invest.* **34**, 1380–1390.
McDonald, D. A. & Taylor, M. G. (1959). *The Hydrodynamics of the Arterial Circulation. Prog. Biophys.* **9**, 105–173. London: Pergamon.
Taylor, M. G. (1959). *Phys. Med. Biol.* **4**, 63–82.

Enzymic removal of adenosine-5′-phosphate (AMP) from substance P extracts. By I. Laszlo. *Department of Pharmacology and Therapeutics, St Thomas' Hospital Medical School, London, S.E.* 1

It has already been reported (Laszlo, 1960) that when substance P was extracted from mouse brain by the method of Amin, Crawford & Gaddum (1954) and assayed on guinea-pig ileum, the amount found varied considerably when different pieces of ileum were used. This discrepancy was traced to the presence of an interfering substance which has been identified as adenosine-5′-phosphate (AMP) by its inhibitory action on rabbit jejunum, by paper chromatography and by its chemical reactions. Extracts prepared by the methods of Leach (1959) and Zetler (1957) were also found to inhibit rabbit jejunum.

Several methods of separating substance P and AMP have been tried, namely, adsorption of substance P by aluminium oxide (Pernow, 1953), precipitation of substance P by ammonium sulphate (Dahlstedt, 1959), paper chromatography in a variety of solvents and by varying the conditions of acetone precipitation from those described by Amin *et al.* None of these methods, however, proved satisfactory.

It was found that inosine-5′-phosphate, which is formed when AMP is deaminated, did not interfere with the assay of substance P on guinea-pig ileum. This suggested the possibility of eliminating the action of AMP by converting it enzymically into inosine-5′-phosphate.

AMP-deaminase was extracted from rabbit muscle by the method of Nikiforuk (1956) and the activity of the enzyme assessed using either ultra-violet absorptiometric estimation of AMP or by the estimation of the liberated ammonia (Russel, 1944). When the hydrochloric acid extract of acetone-dried mouse brain powder was incubated at pH 6·1 with the enzyme preparation, its inhibitory effect on rabbit jejunum, due to AMP, was abolished. After experiments with a number of buffers, maleate was chosen because it was most suitable for the deamination reaction and did not interfere in the subsequent assay. The activity of a solution of substance P was not reduced by incubation with the enzyme preparation.

The enzymic removal of AMP appears to provide a basis for a reliable and relatively simple method for estimating substance P in tissue extract containing this compound.

REFERENCES

Amin, A. H., Crawford, T. B. B. & Gaddum, J. H. (1954). *J. Physiol.* **126**, 596.
Dahlstedt, E., v. Euler, U. S., Lishajko, F. & Östlund, E. (1959). *Acta physiol. scand.* **47**, 124.
Laszlo, I. (1960). *Communication to the British Pharmacological Society.*
Leach, E. (1959). *J. Physiol.* **149**, 34*P*.
Nikiforuk, G. & Colowick, S. P. (1956). *J. biol. Chem.* **219**, 119.
Pernow, B. (1953). *Acta physiol. scand.* **29**, Suppl. 105.
Russel, J. A. (1944). *J. biol. Chem.* **156**, 457.
Zetler, G. & Ohnesorge, G. (1957). *Arch. exp. Path. Pharmak.* **231**, 299.

Inhibition of the acid response to urecholin by antral acidification in dogs. By Mary Redford and B. Schofield. *Department of Physiology, Medical School, King's College, Newcastle upon Tyne* 1

The view that low pH in the pyloric antrum inhibits locally the release of gastrin is at present widely accepted (Longhi, Greenlee, Bravo, Guerrero & Dragstedt, 1958). In most investigations of this effect, gastrin-releasing stimuli have been applied to the mucosal surface of the antrum. It is of interest to consider the effectiveness of the inhibition against stimuli originating on the opposite side of the mucosa. Vagal release of gastrin has been shown to be strongly inhibited by antral acidification by Maung Pe Thein & Schofield (1959). Stable choline esters exert a high proportion of their effect on acid secretion by the gastrin mechanism (Langlois & Grossman, 1950) and represent a blood-borne excitant presumably acting in a similar fashion to vagal excitation. Kim (1955) reported briefly that the

secretion produced by methacholine was inhibited by antral acidification, but his data have not been published.

Four dogs with Heidenhain pouches and pouches of the pyloric antrum were used. The total acid secretion over 1 hr following 0·25 mg urecholin (carbaminoyl β methylcholine chloride) intravenously was compared in experiments in which the antrum was irrigated with 0·05 or 0·1 N-HCl with controls in which it was irrigated with 0·9 % saline. Burstall & Schofield (1954) showed that with this procedure 50–80 % of the acid response was through the antral mechanism. Although day-to-day variation in both control and inhibited responses was considerable, there was highly significant inhibition in three of the dogs. In the fourth, however, the inhibition was not significant. A two-dose procedure was therefore applied in this animal, the response to the second being expressed as $\pm$ a percentage of the first. Three control experiments showed a mean percentage difference of $+39 \pm 14{\cdot}2$ %, and three in which the antrum was acidified during the time of the second response a mean percentage difference of $-39 \pm 13{\cdot}3$ %. This difference is significant, $t = 4{\cdot}0$, $P < 0{\cdot}02$. A series of experiments of this type was subsequently carried out on the other dogs giving an over-all mean percentage difference of $+41 \pm 6{\cdot}1$ % (8) for the controls and $-47 \pm 10{\cdot}4$ % (9) for the inhibited experiments. This difference is highly significant, $t = 7{\cdot}3$. The higher figure for the second response in the control series is explained by the potentiating action of parasympathetomimetic drugs on gastric secretion.

It is concluded that Kim's result is confirmed. The over-all level of inhibition is about 60 %. Allowing for the fact that only half to three-quarters of the stimulating effect is mediated through the antrum, this is of the same order as that observed against vagal release of gastrin by sham feeding by Maung Pe Thein & Schofield (1959).

REFERENCES

Burstall, P. A. & Schofield, B. (1954). *J. Physiol.* **123**, 168–186.
Kim, K. S. (1955). *J. Physiol.* **130**, 14*P*.
Langlois, K. J. & Grossman, M. I. (1950). *Amer. J. Physiol.* **163**, 38–40.
Longhi, E. H., Greenlee, H. B., Bravo, J. L., Guerrero, J. D. & Dragstedt, L. R. (1958). *Amer. J. Physiol.* **191**, 64–70.
Maung Pe Thein & Schofield, B. (1959). *J. Physiol.* **148**, 291–305.

The effect of the inverted position on alveolar-capillary diffusion and volumes of the human lung. By Faith Newman and M. L. Thomson. *Department of Applied Physiology, London School of Hygiene and Tropical Medicine, London, W.C.* 1

Change from the erect to the supine position reduces lung volumes and increases diffusing capacity (e.g. Ogilvie, Forster, Blakemore & Morton,

1956). It was thought that the 'inverted position' might lead to still more marked changes in these measurements.

Three young volunteers A. H., P. B. and A. G., of whom A. H. was in the habit of hanging vertically for periods up to 1 hr or longer, were tied by the feet to an Eve's rocking stretcher and inverted to a position 10° from the vertical.

There was a consistent increase in permeability k_{CO} (which is diffusing capacity × pressure of dry gases/lung volume) from standing to the inverted position (mean increase, 57 %, Table 1).

TABLE 1. The effect of postural change on lung volume and permeability

Subject	P.B.					A.G.					A.H.				
	k_{CO} (/min)	T.V.C. (l.)	V.C. (l.)	T.V.C./V.C. (%)	R.V. (l.)	k_{CO} (/min)	T.V.C. (l.)	V.C. (l.)	T.V.C./V.C. (%)	R.V. (l.)	k_{CO} (/min)	T.V.C. (l.)	V.C. (l.)	T.V.C./V.C. (%)	R.V. (l.)
Standing	{4·61 {4·31	5·05	6·15	82·1	1·3	4·02	5·10	5·60	91·1	1·7	4·50	4·05	4·60	88·0	1·3
Sitting	5·05	5·00	6·00	83·3	1·2	4·58	5·10	5·50	92·7	1·6	{5·54 {5·25	4·17	4·70	88·7	1·1
Lying supine	5·95	4·33	5·31	81·5	1·2	5·40	4·74	5·45	87·0	1·6	—	—	—	—	—
Inverted	6·90	3·85	5·10	75·5	1·2	7·16	4·30	5·25	81·9	1·4	6·41	3·75	4·30	87·2	1·4

All volumes given at B.T.P.S.; V.C. = vital capacity; T.V.C. = 1 sec timed vital capacity; R.V. = residual volume.

The vital capacity (V.C.) showed on the average a 10·0 % fall. The 1 sec timed vital capacity (T.V.C.) was more reduced than V.C. so that the ratio T.V.C./V.C., which is an index of ventilatory efficiency, also fell. The residual volume also tended to fall.

Uniformity of ventilation by modified single-breath oxygen test in P.B. and A.G. was not significantly affected by posture. Pulse rate was the same in sitting and inverted positions.

Subject A.H. remained in the inverted position for 53 min during which k_{CO} fell from 6·41 to 5·64/min. Falls were also shown by P.B. and A.G. after 14 and 18 min.

Using $C^{15}O$, Dollery, Dyson & Sinclair (1960) have shown that exercise is associated with increased perfusion of the upper lobes and a rise in diffusing capacity. The postural effect on diffusion described above may have been due to increase, induced by gravity, in perfusion of the upper lobes, leading to greater area of blood-gas interface. The observed fall in vital capacity would accentuate this increase in k_{CO} by decreasing the volume: surface-area ratio.

REFERENCES

DOLLERY, C. C., DYSON, N. A. & SINCLAIR, J. D. (1960). *J. appl. Physiol.* (in the Press).

OGILVIE, C. M., FORSTER, R. E., BLAKEMORE, W. S. & MORTON, J. S. (1956). *J. clin. Invest.* **36**, 1–17.

Cutaneous haemostasis in the dog's hind limb after perfusion with venous blood. By J. R. MAGALHÃES.* *Instituto Oswaldo Cruz, Rio de Janeiro, Brazil*

Bleeding times following superficial cutaneous incisions were studied in the perfused dog's hind limb under pentobarbitone anaesthesia. The original preparation (Cruz & Oliveira, 1958) was modified so that the limb could be connected to the dog's own circulatory system ('direct' circulation) or perfused with heparinized or citrated donor dogs' blood by a pump. During 'direct' circulation no anticoagulants were used. Bleeding times were within the normal range (5–12 min) during 'direct' circulation, or when perfusion was done with arterial blood. Perfusion with venous blood 'reoxygenated' *in vitro* prolonged the bleeding time to more than 30 min. Subsequent perfusion with arterial blood did not reverse this prolongation of the bleeding time, confirming Cruz, Oliveira & Magalhães (1958).

It has been found in the present series of experiments that when 'direct' circulation was re-established, normal bleeding times were again attained.

Heparinization of the animal (320 i.u./kg body weight) at this stage did not lead to an increase of bleeding time, showing that in this preparation normal bleeding times can occur in the absence of blood coagulability.

REFERENCES

CRUZ, W. O. & OLIVEIRA, A. C. (1958). *J. appl. Physiol.* **13**, 368–374.
CRUZ, W. O., OLIVEIRA, A. C. & MAGALHÃES, J. R. (1958). *J. Physiol.* **142**, 242–250.

Is increased vasomotor tone present in hypertension? By J. CONWAY. *Charing Cross Hospital Medical School, London, W.C. 2*

Brief anoxia following rapid decompression from 560 to 140 mm Hg absolute (8,000–40,000 ft.; 2·44–12·2 km) and its prevention. By J. ERNSTING and G. J. R. MCHARDY. *R.A.F. Institute of Aviation Medicine, Farnborough, Hants.*

Concentrating activity of the gall bladder. By J. M. DIAMOND. *Physiology Laboratory, University of Cambridge*

The mechanism of the external secretion of the pancreas. By S. DIKSTEIN. *Department of Pharmacology, Medical School, Hebrew University, Jerusalem, Israel*

* British Council Scholar. Present address: Royal Free Hospital School of Medicine, London, W.C. 1.

Notice to Contributors. Papers submitted for publication should be sent to:

THE EDITORS OF THE JOURNAL OF PHYSIOLOGY

Physiology Department, The Medical College of St Bartholomew's Hospital,

Charterhouse Square, London, E.C. 1

Contributors should follow the instructions in **'Suggestions to Authors'** with regard to the preparation of papers, and should study carefully the format of papers in recent numbers of the Journal. The latest edition of this pamphlet appeared in Vol. 150, p. 1. Offprints may be obtained from **The Cambridge University Press, 200 Euston Road, London, N.W. 1.** Price 2*s.* in paper cover, post free.

Papers submitted for publication should be accompanied by a declaration in the following terms: The attached paper entitled ..... has not been, and is not intended to be published anywhere except in the Journal of Physiology, and we/I agree that, if and when it is accepted by the Editors for publication, in consideration for such acceptance the entire copyright in this paper shall pass thereupon to the Physiological Society.

Requests for permission to reproduce any part of papers published in the Journal of Physiology should be addressed to the Secretary of the Editorial Board at the Physiological Laboratory, Cambridge. The Editorial Board will normally grant all requests from authors to reproduce material from their own papers. Permission will not be given to third parties except with the consent of the authors concerned, but if the Editorial Board receives no reply within a reasonable period of writing to the authors at their last address notified, consent will be assumed.

When a paper is submitted by several authors in collaboration their names will be printed in alphabetical order.

The date of receipt printed at the head of each paper is normally the date on which the typescript is first received by the Editors, but if a paper has to be extensively altered after submission the date may at the discretion of the Editors be changed to that on which an acceptable draft is received.

Authors are reminded that the acceptance of papers may be prevented by their being faulty in English composition. It is also essential that papers should be written as concisely as possible, without undue repetition of material in text, tables and figures. Details under the heading 'Methods' will appear in small print unless the author particularly wishes otherwise. Papers concerned with methods only are not acceptable, unless the methods involve new principles and are shown to be of proved value and interest to physiologists. Long theoretical discussions are in general unsuitable.

In an exceptional case the Editors will be prepared to accept a coloured illustration if they consider it to be necessary for conveying the scientific content of the paper. Half the cost of reproduction will be charged to the author.

Authors are requested to verify that the wording of references to unpublished work, and of acknowledgements of help or criticism, is approved by the persons concerned.

The Editors cannot accept responsibility for damage to or loss of papers submitted to them. Contributors are advised to keep copies of their typescripts and illustrations.

Proofs should be corrected and returned promptly. Unless otherwise directed, proofs are sent to the author whose name is first alphabetically, at the address from which the paper originates. If the author(s) wish the proofs to be sent to any other person, or to any other address, instructions should be given.

Abstract. Authors should send with their typescript an abstract suitable for inclusion in *International Abstracts of Biological Sciences*. This abstract will not appear in the *Journal of Physiology* but will be scrutinized by the Editors before being passed for publication in *International Abstracts*. It should contain a brief outline of the results and definitive conclusions of the work. Details of methods are generally not required. A paper of average length should be abstracted in about 100 words. The abstract should be typed in double spacing on a separate quarto sheet, and should be headed as follows: Title of paper; name(s) of author(s); *J. Physiol., Lond.*, followed by space for year, volume and page reference; abbreviated postal address including country of origin. For example:

A quantitative description of membrane current and its application to conduction and excitation in nerve. A. L. Hodgkin and A. F. Huxley. *J. Physiol., Lond.*, 1952, **117**, 500–544 (Physiol. Lab., Univ. Camb., Cambridge, England).—Text of abstract follows straight on.

The short title *J. Physiol., Lond.*, is used in the abstract to avoid confusion with *J. Physiol., Paris*, but should not be employed in the reference lists of papers submitted to the Journal.

THE BIOCHEMICAL JOURNAL

Vol. 76, No. 3 **CONTENTS** *September 1960*

Effect of dietary fat intake and age on polyunsaturated fatty acids in human-blood serum. By V. S. PATIL and N. G. MAGAR.

Serum-cholinesterase activity in health and in liver disease. By R. G. O. KEKWICK.

The biochemistry of the nitrifying organisms. 6. The effect of oxygen concentration on nitrite oxidation in the presence of different inorganic ions. By W. D. BUTT and H. LEES.

The pyridine nucleotide metabolism of mitochondria incubated with and without added substrates and metabolic inhibitors. By L. M. BIRT and W. BARTLEY.

Exchange of potassium ions across a concentration difference by isolated rat-liver mitochondria. By J. E. AMOORE.

The biochemistry of rumen protozoa. 3. The carbohydrate metabolism of *Entodinium*. By A. R. ABOU AKKADA and B. H. HOWARD.

Some properties of neutral-salt-soluble collagen. 1. By J. H. FESSLER.

Some properties of neutral-salt-soluble collagen. 2. By J. H. FESSLER.

The phospholipids of human-blood plasma and their exchange with the cells. By C. E. ROWE.

Metabolic heterogeneity of human γ-globulin. By S. COHEN and T. FREEMAN.

The lamellibranch crystalline style. By K. BAILEY and B. D. WORBOYS.

Carbohydrate metabolism in *Spirochaeta recurrentis*. 1. The metabolism of spirochaetes *in vivo* and *in vitro*. By J. D. FULTON and P. J. C. SMITH.

Carbohydrate metabolism in *Spirochaeta recurrentis*. 2. Enzymes associated with disintegrated cells and extracts of spirochaetes. By P. J. C. SMITH.

Carbohydrate metabolism in *Spirochaeta recurrentis*. 3. Properties of aldolase in spirochaetes. By P. J. C. SMITH.

Carbohydrate metabolism in *Spirochaeta recurrentis*. 4. Some properties of hexokinase and lactic dehydrogenase in spirochaetes. By P. J. C. SMITH.

The biosynthesis of chondroitin sulphate. Incorporation of sulphate into chondroitin sulphate in embryonic chick cartilage. By J. B. ADAMS.

Some observations on the kinetics of the reactions with gases of natural and reconstituted haemoglobins. By E. ANTONINI and Q. H. GIBSON.

Study of a proteolytic enzyme from rabbit spleen. By C. LAPRESLE and T. WEBB.

Effects of the insecticides DDT and dieldrin on phosphorus metabolism of the adult housefly *Musca domestica* L. By F. P. W. WINTERINGHAM, G. C. HELLYER and M. A. MCKAY.

Metabolism in the New Zealand strain of obese mice. By K. SUBRAHMANYAM.

A protein disulphide reductase from pea seeds. By M. D. HATCH and J. F. TURNER.

Studies on vitamin E. 4. The simultaneous determinations of tocopherols, ubiquinones and ubichromenols (substance SC) in animal tissues: a reconsideration of the Keilin–Hartree heart preparation. By A. T. DIPLOCK, J. GREEN, E. E. EDWIN and J. BUNYAN.

Fission products and the dairy cow. 2. Some aspects of the metabolism of the alkaline-earth elements calcium, strontium and barium. By R. J. GARNER, H. G. JONES and B. F. SANSOM.

Uncoupling reagents and metabolism. 1. Effects of salicylate and 2:4-dinitrophenol on the incorporation of ^{14}C from labelled glucose and acetate into the soluble intermediates of isolated rat tissues. By M. J. H. SMITH and V. MOSES.

Uncoupling reagents and metabolism. 2. Effects of 2:4-dinitrophenol and salicylate on glucose metabolism in baker's yeast. By V. MOSES and M. J. SMITH.

Studies in detoxication. 82. The fate of benzoic acid in some domestic and other birds. By B. C. BALDWIN, D. ROBINSON and R. T. WILLIAMS.

Studies in detoxication. 83. The aromatization of *cyclo*hexanecarboxylic acid in hens. By B. C. BALDWIN, D. ROBINSON and R. T. WILLIAMS.

The formation *in vivo* of lombricine in the earthworm (*Megascolides cameroni*). By R. J. ROSSITER, T. J. GAFFNEY, H. ROSENBERG and A. H. ENNOR.

Structure of the ribitol teichoic acid from the walls of *Bacillus subtilis*. By J. J. ARMSTRONG, J. BADDILEY and J. G. BUCHANAN.

Quantitative studies of the avidity of naturally occurring substances for trace metals. 4. A potentiometric method for highly insoluble complexes, and its application to complexes of adenine anion. By A. ALBERT and E. P. SERJEANT.

Proceedings of the Biochemical Society.

Subscription price per volume 80s. net. $13.50 in U.S.A.

CAMBRIDGE UNIVERSITY PRESS

BENTLEY HOUSE, 200 EUSTON ROAD, N.W.1

BLOOD FLOW IN ARTERIES

DONALD A. McDONALD

Reader in Physiology, St Bartholomew's Hospital, University of London

— the Seventh of the Monographs of the Physiological Society.

Contents include the nature of flow in a liquid, the characteristics of blood flow in the circulation, the disturbances in blood flow due to the configuration of the vascular bed, the pressure-flow relationship of oscillatory flow, the measurement of phasic flow velocity, the pulsatile flow pattern in arteries and the physical properties of the arterial wall.

320 pages 70 illustrations
40s. net

EDWARD ARNOLD

Particulars of the

JOURNALS

Published by the Cambridge University Press, may be had from

THE MANAGER
CAMBRIDGE UNIVERSITY PRESS
BENTLEY HOUSE
200 EUSTON ROAD
LONDON, N.W. I

THE BRITISH JOURNAL OF NUTRITION

PUBLISHED FOR THE NUTRITION SOCIETY

Vol. 14, No. 4 Shortened Versions of Titles 1960 35s. net

Obituary notice (Major-General Robert McCarrison, K.). By W. R. AYKROYD.

Low-protein diet and serum proteins of kittens. By BETTY L. COLES.

Nutritive value of proteins of Bengal grams. By G. C. ESH, T. S. DE and U. P. BASU.

Effect of prolonged feeding of differently saturated fats to laying hens. By HANS FISHER, H. S. WEISS, G. A. LEVEILLE, A. S. FEIGENBAUM, S. HURWITZ, O. DONIS and H. LUTZ.

Degradation of protein in the rumen of sheep. By T. H. BLACKBURN and N. P. HOBSON.

Severe undernutrition in growing and adult animals. 4. By ELSIE M. WIDDOWSON, J. W. T. DICKERSON and R. A. MCCANCE.

Ubiquinone in rats deficient in vitamin A. By T. MOORE and I. M. SHARMAN.

A microbiological method for assessing the nutritional value of proteins. By J. E. FORD.

Antibiotic, copper and zinc supplements for growing pigs. By R. S. BARBER, R. BRAUDE and K. G. MITCHELL.

Severe undernutrition in growing and adult animals. 5. By R. A. MCCANCE and L. R. MOUNT.

Use of antipyrene and *N*-acetyl-4-aminoantipyrene in the determination of body water in cattle. By F. WHITING, C. C. BALCH and R. C. CAMPLING.

Combined Subscription for The British Journal of Nutrition (4 issues) and The Proceedings of the Nutrition Society (2 issues) is £7 per annum.

Cambridge University Press, Bentley House, 200 Euston Road, London, N.W.1

THE JOURNAL OF PHYSIOLOGY

★ The subscription price, for orders placed in advance of publication, for vols. 153, 154 (1960) is 80*s.* per volume of 3 parts post free ($13.50 in the U.S.A.).

In normal circumstances, five volumes will appear in each year.

Orders may be placed with your Bookseller, or sent direct to the **Cambridge University Press, Bentley House, 200 Euston Road, London, N.W. 1.**

★ Also available from the **Cambridge University Press**

Back numbers from vol. 61 (1926) onwards.
Volumes 61–115, price £4. 10*s.* per volume.
Volumes 116–120, price £4 per volume.
Volumes 121–130, price £3. 15*s.* per volume.
Volumes 131–152, price £4. 10*s.* per volume.
Single issues and complete sets can be supplied.

Author and subject indexes to the Journal of Physiology, prepared by Dr Grace Eggleton.
Vols. 61–100. 30*s.* net.
Vols. 101–115. 30*s.* net.

★ Available from **Wm. Dawson & Sons, Ltd. 4 Duke Street, Manchester Square, London, W. 1**

Back numbers, vols. 1–25. Price £6 per volume.
Members' rates on application.

Some back issues and volumes are available from Volumes 1–60, particulars on request.

Author index of the Journal of Physiology, vols. 1–60. 25*s.* net.

Subject index of the Journal of Physiology, vols. 1–60, prepared by Dr J. G. Priestley. 15*s.* net.

History of the Physiological Society during its first fifty years 1876–1926. By Sir Edward Sharpey-Schafer, F.R.S. 15*s.* net.

Journal of Physiology, Volumes 26–60

Permission has been granted by THE PHYSIOLOGICAL SOCIETY for the reprinting of the above. An announcement giving date of publication will be made in a subsequent issue. Librarians and others wishing to have copies of these volumes may place their reservations now. All enquiries should be sent to

Wm. Dawson & Sons Ltd., 4 Duke Street, Manchester Square, London, W. 1

who are undertaking this Reprint.